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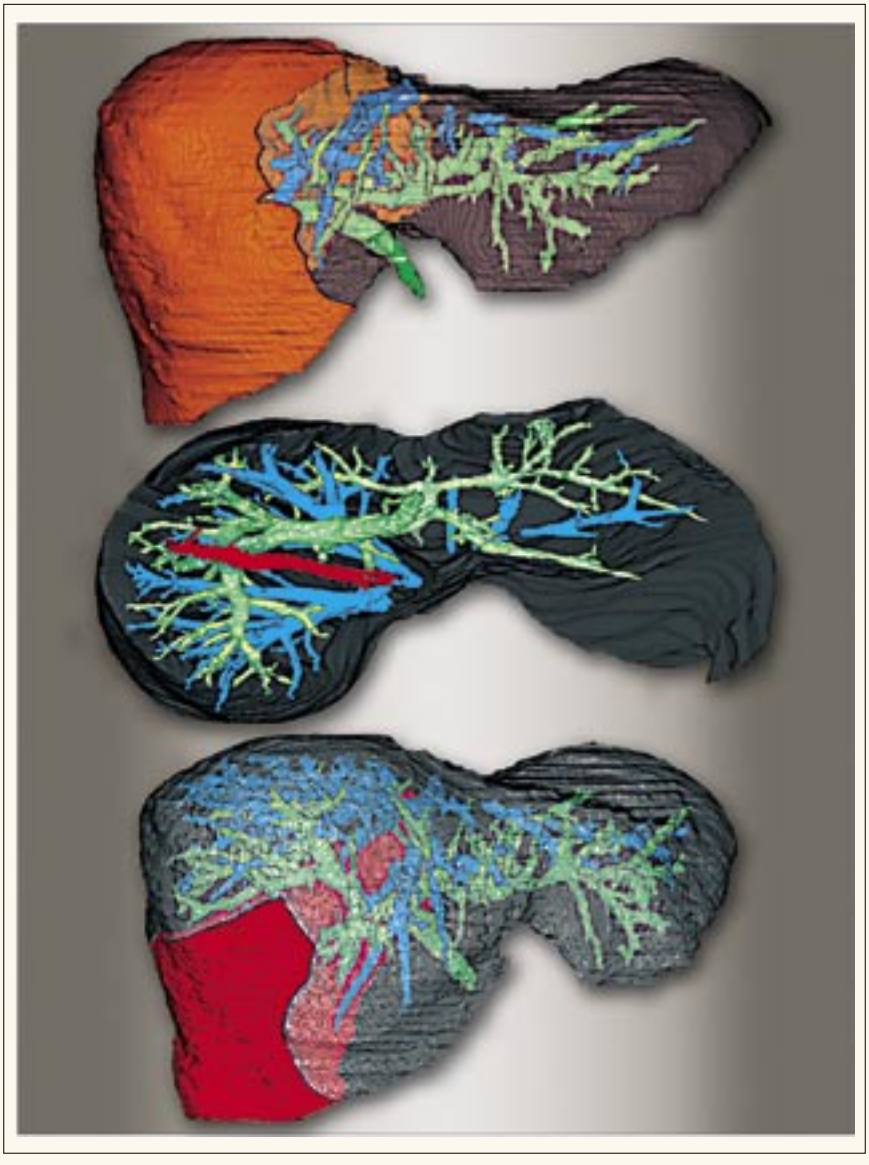

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Cover Illustration: Hepatectomy simulation for LDLT with right lobe graft. (A) For preoperative volumetry of the right lobe graft (shown in orange), the right portal pedicle was clipped. (B) Integrated 3D axial view showed IRHV (shown in magenta). Liver was observed from caudal to cranial direction. (C) Drainage area by IRHV was shown in red color and its estimated volume was 234 mL, which accounted for 36% of proposed right lobe graft (from [73]); Fujimoto J, Yamanaka J. Liver resection and transplantation using a novel 3D hepatectomy simulation system, Advances in Medical Sciences, 2006; 51: 7-14.

Liver resection and transplantation using a novel 3D hepatectomy simulation system

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Abstract

In liver surgery, accurate assessments of liver resection volume and anatomical variation are mandatory for preoperative planning of safe curative hepatectomy. In living donor liver transplantation (LDLT), estimation of hepatic venous drainage is important to avoid liver graft and donor residual liver congestion. This paper reviews the articles on simulation-guided liver surgery and describes our novel 3D hepatectomy simulation system for liver resection and transplantation. Our 3D simulation system, based on the hepatic circulation, provided accurate volumetric and stereotactic information for preoperative planning of curative hepatectomy. In addition, our simulation program was applicable to the hepatic venous system to predict liver congestion in LDLT. Future studies include assessment of the impact of the simulation technologies on surgical education, and their exact cost-effectiveness must be also assessed objectively.

Key words: simulation, 3D CT, hepatectomy, living donor liver transplantation, liver congestion.

Introduction

Surgery demands a significant amount of cognitive analysis and integration of enormous patient data. Surgeons have always been confronted by a difficulty in understanding three-dimensional (3D) image from two-dimensional (2D) information obtained by preoperative radiological investigation. The possibility to overcome limitation of the cognitive ability was sought with the advent of high-performance computer technology. Marsh et al. [1] reported an initial experience of 3D computer simulation in the field of craniofacial surgery. Computer-aided simulation was applied for treatment planning of radiotherapy [2], neurosurgery [3], and orthopedic surgery [4] in tern.

Particularly in the field of hepatic surgery, imagination of the 3D image from the 2D computer tomography (CT) or magnetic resonance (MR) images is difficult because of the anatomical complexity and hepatic vascular variability. Intraoperative ultrasonography (IOUS) has been used to determine tumor location and to serve as a guide for hepatectomy [5]. However, there was still a difficulty in reconstructing a 3D image of the tumor and adjacent blood vessels only by the 2D information of IOUS. Despite the availability of 3D ultrasound probes for abdominal sonography, ultrasound as a means of 3D reconstruction has not proven successful because of optical distortions and low contrast behavior of the visualized lesions. The reproducibility of detected images also depends on the skill of the examiner. In contrast, innovations in CT and MR technology over the last two decades considerably improved resolution and scanning time.

In 1991, Hashimoto et al. [6] reported development of a 3D image reconstruction for hepatic anatomy. With the advent of the 3D rendering technique, improved preoperative determination of the tumor location within a liver subsegment was reported [7]. Significant anatomic variations in the segmental anatomy of the liver were also recognized using the 3D CT images [8-10]. Thus the usefulness of the 3D CT has been reported to provide detailed hepatic segmental and vascular anatomy [11-13]. In addition to the detailed topography, there are other important aspects for liver surgery.

Primary hepatocellular carcinoma (HCC) and metastatic liver cancer are the representative and refractory hepatic malignancies requiring surgical resection [14,15]. Majority of HCC have chronic liver disease and associated impaired hepatic function restricts the extent of hepatectomy. The need for living donor liver transplantation (LDLT) is also increasing because of the evolving indication including HCC cases and a shortage of

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decreased organ donation [16,17]. In LDLT, an insufficient graft may jeopardize recipient survival and an excessive liver resection may cause donor liver failure. Therefore, accurate estimations of liver resection volume and residual liver volume are mandatory for successful liver resection, donor hepatectomy, and recipient transplant operation [18,19].

Conventional planimetry has been used to estimate the resected and residual liver volumes, although considerable volumetric error has been observed [20-22]. The calculations are performed by manually tracing around the margins of the hepatic parenchyma on each 2D CT axial image using an electronic cursor. The cross-sectional area (cm^2) within the presumed liver sectors between the hepatic veins is determined, and all individual areas are summed, yielding the liver volume of the interested region (cm^3). However, this method does not account for hepatic vascular perfusion. Moreover, prediction of segmental liver volume has been impossible.

Liver resection currently offers the only potential cure for HCC and metastatic liver cancer when the resection margin is negative [23,24]. Hence, preoperative accurate assessment of the resection margin is also important to achieve curative hepatectomy [25,26]. The other important issue in LDLT includes estimation of the hepatic venous drainage to avoid liver graft and donor residual liver congestion [27]. The preoperative assessment of the hepatic vein drainage should be available to decide necessity of hepatic vein branch reconstruction.

Several studies have reported use of 3D CT as an operation planning system for liver surgery [28,29]. These pioneering studies, however, did not refer to a predictive function of the liver resection volume or resection margin. Moreover, the predicted parameters were not validated by the comparison to the actual resected specimens. Here we review the articles on simulation-guided liver surgery and describe our novel 3D hepatectomy simulation system for liver resection and transplantation.

Virtual reality and simulation

Virtual reality implies a 3D computer-generated world that mimics the real world and allows participants to interact with and navigate it. Virtual reality is created from converting a 2D image into 3D numeric data thus creating a virtual image. The term 'virtuality' was introduced by Lanier [30] in 1989, although its development dates back to the early 1960s, when the first graphic computers were built. Physicians and surgeons first encountered computer-generated images with the development of CT, US, and MR in the late 1970s. These devices dramatically changed the practice of medicine only in a decade. The word 'simulation' was first brought into the literature of plastic surgery in 1985, when Marsh et al. [1] described the term 'surgical simulation'. Simulation can be defined as a device or exercise that enables the participant to reproduce or represent, under test conditions, phenomena that are likely to occur in actual performance. During the 1990s, 3D image reconstruction became possible and the first surgical simulators appeared, starting with tendon transfer model [31] and abdominal surgery simulator [32]. The 3D visualization of images facilitates the visibility of their content and allows three new ways of perception: immersion, navigation, and interaction [33,34]. Virtual reality is particularly relevant to the analysis of the stereoscopic relationship between a tumor to be resected and the vascular anatomy of the liver for the planning of hepatic resections. The proposal for the reasonable compromise between the radicality and the hepatic damage can be optimized preoperatively.

Liver surgery simulation

There are three reasons why a hepatic surgery simulation is required. The first, is to provide the surgeon with a comprehensive visualization of the liver organ, allowing accurate presurgical localization of the pathological lesion and perception of its relation with vascular and biliary system. This step allows the surgeon to plan the best surgical approach. The second reason, is to allow planning and realistic surgical simulation, such as the detailed flight plan used by jet pilots. The surgeon will be able to practice a given procedure repeatedly and be better prepared for the intervention in the surgical conditions. The third reason, is that virtual reality is an integral part of computer-assisted surgical procedures. Augmented reality will superimpose the virtual image of the hepatic vessels and tumor onto the preplanned resection plane to create the real operating view. The surgeon will have precise knowledge about the position of crucial anatomical landmarks that were formerly unseen.

Couinaud's liver segment model

Since 1954, Couinaud's liver segment classification has become the standard basis for liver surgery [35,36]. Because the portal, arterial, biliary, and lymphatic systems are grouped together in the vasculobiliary sheaths, their intrahepatic ramification pattern corresponds in detail, and the portal branching pattern is indicative of the intrahepatic segmentation [37]. Couinaud defined avascular planes within the liver separating autonomously functioning units. Despite the reliance of surgeons on the Couinaud's classification system, increasing suspicion emerged about the segment borders, especially against the background of living-donor surgery. The position and shape of the segment borders are variable, and are hidden within the homogeneous liver mass.

Estimation of liver volume and conventional planimetry

Historically, anthropometric and radiological methods have been used for measuring liver volumes. Anthropometric data to estimate liver volume are based on height, weight, body surface area, age, and gender [38,39]. Variability due to overall body habitus, particularly the effect of obesity, gender, and racial differences, has limited their value. In addition, the anthropometric method does not allow for the differences in lobar volumes: the right liver has been shown to vary between 49% and 82% of total liver volulme [40]. Radiological methods have shown some improvement in liver volume estimation compared with anthro-

pometric data. Early studies of liver volume measurement with CT scan traced serial 1-cm liver slices and summated them: day-to-day variability was ±6%, and interobserver variability ±5%. These calculations are performed by manually tracing around the margins of the hepatic parenchyma on each CT image using an electronic cursor. The cross-sectional area (cm^2) within the region of interest is determined, and all individual areas are summed, yielding the total liver volume (cm^3). However, considerable volumetric error has been observed by the electronic planimetry for the estimation of the resected and residual liver volumes [20-22]. In addition, the conventional method did not allow estimates of sectorial or segmental liver volume.

Development of computer-aided surgery system

Reconstruction of the liver, vessels, and tumor images using CT and MR slice data was initially reported for simulation of laser coagulation therapy of metastatic liver cancer [6]. The 3D CT assessment using arterial portography (CTAP) was useful for more accurate segmental or subsegmental location of hepatic metastases than the 2D CTAP preoperatively [11,41]. The advent of the volume rendering technique allowed simultaneous 3D display of the liver parenchyma, tumor, and vessels. By assigning a low opacity to the liver parenchyma and a high opacity to the tumor and vessels, visualization of the tumor location within the liver capsule became possible and the tumor position relative to the vascular anatomy was appreciated [42]. Zahlten et al. [43] reported a region growing technique for extraction of the 3D portal vein image from CT data. Using cadaver cross-sectional data, Marescaux et al. [33] showed potential application of 3D hepatic visualization to virtual reality concepts and surgical planning. In 2000, a pioneering concept of automatic segmentation of the hepatic vascular system were reported for liver surgery planning [12,28]. Preoperative 3D CT was used for more detailed depiction of the portal vein branch such as the caudate branch, facilitating caudate lobectomy and the selection of the interlobar plane for transection in the transhepatic anterior approach [44]. Moreover, in a clinical trial involving 27 patients scheduled for liver surgery, 3D reconstruction of CTAP image was applied to volumetric estimation of hemi-liver resections [13]. However, this method did not account for hepatic vascular perfusion and was not able to predict liver volume at Couinaud's segment level.

Operation planning system

Lamade et al. [28] evaluated the impact of 3D presentations on the operation planning. The 2D and 3D liver images of 7 virtual patients were presented to a total of 81 surgeons at different levels of training. The precision of the tumor assignment to a liver segment and resection proposal was assessed. The liver segment determination was significantly correlated to the level of training. There was a significant increase in the precision of tumor localization and resection proposal with 3D reconstruction compared with 2D reconstruction. Lang et al. [45] reported 7 cases, in whom the results of computer-associated risk analysis led to a change of operation planning with regard to the extent of resection (n=3) or the need for vascular reconstruction (n=4). In their study, resections most likely to leave devascularized segments were the extended left hepatectomy combined with wedge resection of the right lobe. This was explained by the variability of hepatic vascular system of the right and middle lobes.

Simulation for living donor liver transplantation

Living donor liver transplantation (LDLT) in the adults allows healthy adults to donate a portion of their liver to compatible recipients [46-48]. Right lobe hepatectomy should not endanger the vascular supply or metabolic function of the remaining left lobe of the healthy donor. An excessive liver resection may result in donor liver failure. Sufficient left lobe liver volume must be preserved to permit metabolic function during regeneration. Liver remnant volume of 30-40% of the total liver volume is necessary for the donor to survive, provided that the liver parenchyma is normal without evidence of fatty infiltration [49]. In contrast, a small-for-size graft may result in malfunction or may not sustain metabolic demand in the recipient. Small-for-size grafts are prone to dysfunction, not only because of the insufficient functional hepatic mass but also because of the excessive portal perfusion adversely affecting graft and sinusoidal cells [50]. The minimum graft volume required to provide sufficient functional hepatocytes to the recipient is approximately 40% of the standard liver volume, as calculated using the body surface area [51]. Total liver volume is reported to have a relatively constant relation to body weight, ranging between 2-2.7% in healthy subjects [52]. However, the right and left lobe volumes are widely variable [18]. Moreover, anatomical complexity and variability in hepatic vessels make donor hepatectomy difficult procedure. Therefore, exact preoperative information on detailed topography and precise liver graft volume should be obtained for the preoperative planning of safe donor hepatectomy and successful LDLT [18,53,54].

Application of MDCT

The use of multidetector technology has dramatically increased the speed of data acquisition, resulting in thin-slice images and decreased motion artifacts, compared with the conventional scanners. The thin-slice axial images allow accurate 3D reconstructions of the liver and depiction of the shape of the graft. The usefulness of 3D CT has been reported for the preoperative assessment in LDLT [55,56]. Selecting living adult donors has to be performed with the utmost precision, as the donor hepatectomy has to be performed with zero mortality. Exact volumetric prediction of the transplant liver lobe and residual liver lobe is important for selection of the donor. Kamel et al. [56] reported accurate and reproducible lobar volume determination by virtual right hemihepatectomy using 3D MDCT. In addition, the complex vascular anatomy of the

liver and the high incidence of vascular variants reinforced the need for accurate preoperative vascular imaging. Variations in hepatic arterial anatomy, hepatic venous anatomy, and portal vein anatomy were reported in approximately 45% [57], 30% [58], and 20% [59] of patients, respectively. Portal vein variation such as absence of the right portal vein trunk is considered contraindication to living donor operation [55]. A higher spatial resolution of MDCT compared to MR angiography allowed more accurate and reliable display of the hepatic arterial system with a higher number of detected variants and a higher image quality [60]. The use of "all-in-one" MDCT technology also enabled display of biliary structures facilitating the transplant operation planning process and will be discussed later.

Venous drainage and congestion in LDLT

Adult LDLT requires a right lobe graft for adequate liver volume. However, a right lobe graft without a middle hepatic vein (MHV) potentially has problems of hepatic venous congestion, which is caused by absence of drainage via MHV tributaries (V5 and V8) or the inferior right hepatic vein (IRHV) [61,62]. Preoperative prediction of the congestion volume has been difficult. A large variability in the hepatic vein anatomy has been reported [63,64]. The inferior right hepatic vein was reported with a frequency of 6-29% [65,66]. The problem is that demarcation line of the hepatic venous congestion becomes evident only after parenchymal transection and temporary arterial clamping of the donor liver [27]. The importance of optimal venous outflow for sufficient liver function has become evident with the development of right lobe LDLT [67,68]. Venous congestion would result in functional impairment or even necrosis, predisposing to biliary and infectious complications. Generally, T2-weighted MR shows that the water component of tissue and increased signal intensity is consistent with the presence of tissue congestion in solid organs [69]. Using MR congestion score, Yamamoto et al. [70] showed that MR changes compatible with tissue congestion occurred in 80% to 90% of the grafts. There was also a correlation between the graft congestion and posttransplant ascites. In LDLT using the right lobe, IRHV with a diameter of 5 mm or more has been believed to require reconstruction. However, no quantifiable criteria for the venous reconstruction have been available to avoid liver congestion. The volumetric estimation of the hepatic venous drainage is required to avoid liver graft and donor residual liver congestion.

Novel 3D hepatectomy simulation

In order to create an operating system available for the clinical application in liver surgery, an automatic image-processing system of 3D liver reconstruction from CT images has been developed [71]. Our novel 3D image processing software (Hitachi Image Processing System, Version 0.7a) was developed by the Department of Radiology, Hyogo College of Medicine, Nishinomiya, Japan and Hitachi Medical Corporation, Tokyo, Japan. The simulation system was implemented as a plug-in in the portable PC, which is convenient to carry between the operating room and the office. The 3D images of the tumor, portal vein, hepatic vein, and liver parenchyma were reconstructed using the transferred CT image data by region growing technique. A transparent display employed in this system provides perspective views of the liver. Rotation, cross-sectioning, and enlargement functions allow detailed understanding of the anatomic structure between the hepatic vessels and the tumor preoperatively. The simulation software introduced an algorithm to define the perfusion area of individual portal vein branches according to the direction and diameter of the vessels. Computation of the perfusion area continued along the individual portal branches proximally to peripherally until the entire liver parenchyma was subdivided. Thus the vascular perfusion area was calculated by an algorithm based on the direction and diameter of hepatic vessels. The resection planning was proposed by calculation of the liver resection volume based on the vascular perfusion area, and the resection margin.

Table 1. **Clinical characteristics of patients (n=72)**

Mean of age (yr)	62
Sex	
Female	21 (29)
Male	51 (71)
Operative indication	
HCC	57 (79)
Living-related donor	3 (5)
Other malignant tumors	10 (14)
Benign tumors	2 (2)
Surgical procedure	
Trisegmentectomy or more	17 (24)
Bi- or mono-segmentectomy	26 (36)
Limited resection	29 (40)

HCC – hepatocellular carcinoma.
All number in parentheses are percentages unless indicated otherwise (from [71])

Between May 2001 and June 2004, 72 consecutive patients received preoperative hepatectomy simulation at the Hyogo College of Medicine (*Tab. 1*). Computed tomography, which was performed either angiographically or intravenously, provided fundamental information for the preoperative donor hepatectomy simulation. Multidetector CT scan (MDCT) has become routine use since 2004 with a slice thickness of 1 mm. To validate the volumetric accuracy of the simulation system, the predicted liver resection volume was compared to the actual weight of the resected specimen. A significant correlation existed between the simulation predicted liver resection volume and the actual weight of the resected specimen ($r=0.96$, $P<0.0001$) (*Fig. 1A*). The difference between the estimated volume and the actual weight was 9.3 ± 6.0 ml. A significant correlation was also revealed between the predicted and actual resection margins ($r=0.84$, $P<0.01$) (*Fig. 1B*). The difference between the predicted and actual margins was 1.6 ± 2.6 mm. Our simulation system enabled the accurate prediction of the liver resection volume at Couinaud's segment levels. We demonstrated systematic overestimation of liver resection volume by a mean of 9 ml, as compared to previously reported values of

Figure 1. **Validation of liver resection volume and resection margin. (A) Significant correlation existed between simulation predicted liver resection volume and actual weight of resected specimen. (B) Significant correlation also existed between predicted and actual margins (from [71])**

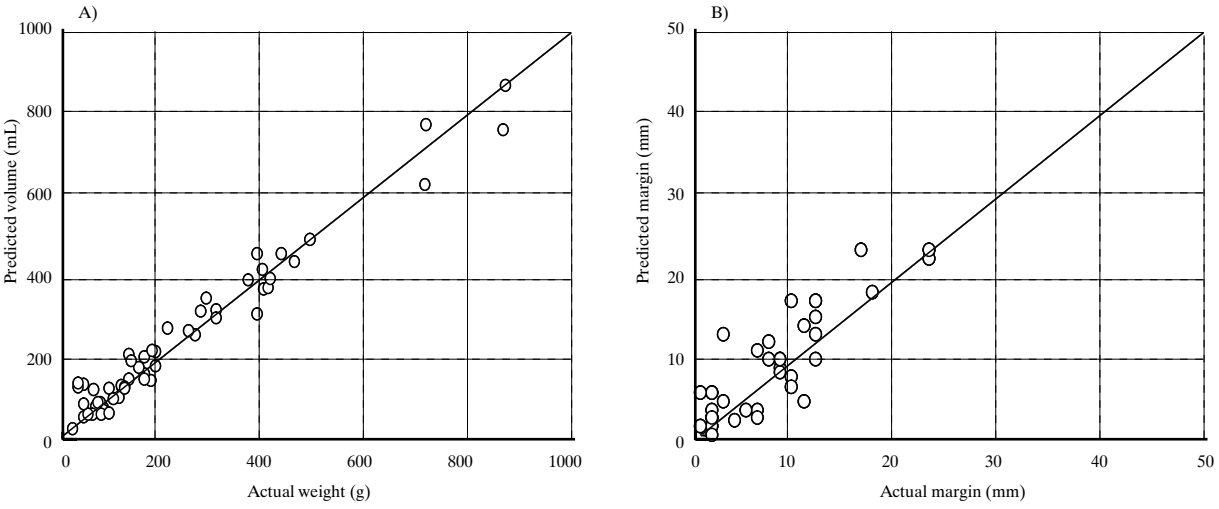
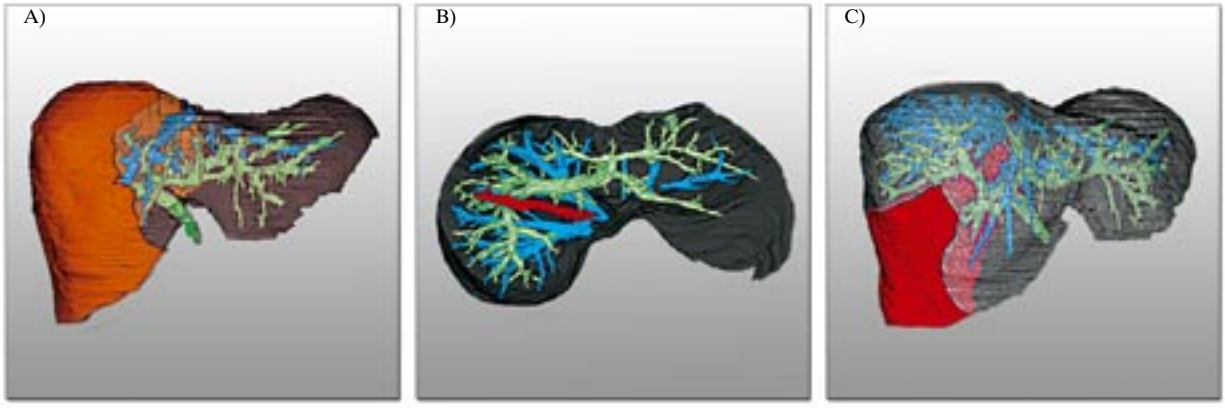


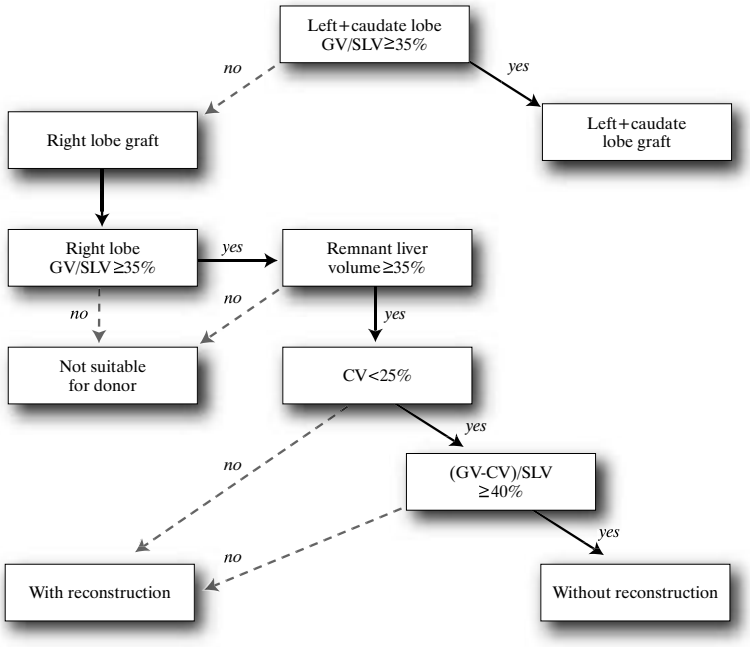
Figure 2. **Hepatectomy simulation for LDLT with right lobe graft. (A) For preoperative volumetry of the right lobe graft, the right portal pedicle was clipped. (B) Integrated 3D axial view showed IRHV. Liver was observed from caudal to cranial direction. (C) Drainage area by IRHV was shown in red color and its estimated volume was 234 mL, which accounted for 36% of proposed right lobe graft (from [73]).**



53 to 90 mL [13,22,72]. The predicted liver resection volume and margins by the simulation correlated significantly with the actual weight and margins of the surgically resected specimens. In our simulation, inclusion of the entire tumor within the predicted resection area allowed the preoperative planning of curative resection.

In cases of LDLT, the simulation algorithm was applied to the hepatic venous system to estimate the drainage area of the hepatic vein by clipping of the corresponding vein at its origin. The preoperative planning for donor hepatectomy was proposed via calculation of the liver graft volume and hepatic vein drainage area. A case of donor hepatectomy for right hemi-liver graft was presented as an example (*Fig. 2*) [73]. Clipping of the right portal pedicle at its origin prompted volumetric calculation of the corresponding portal perfusion area as a predicted graft volume. The predicted right hemi-liver graft volume was 648 mL, with a graft-to-recipient weight ratio of 0.9%. The actual liver graft weighed 640 g. In this case, an inferior right hepatic vein (IRHV) of 8 mm in diameter was identified by the preoperative simulation. Then the simulation algorithm was applied to the hepatic venous system to estimate the drainage area of the hepatic vein branch. The estimated drainage volume by the IRHV was 234 mL and accounted for 36% of the proposed graft. Based on the volumetric calculation of the hepatic vein drainage area, the reconstruction of IRHV and RHV was necessary to avoid congestion of the implanted liver graft. The recipient recovered uneventfully and the follow-up dynamic CT scan revealed patent IRHV and RHV without evidence of congestion. None of the previous studies used the volumetric assessment of the congested areas as a criterion for the venous reconstruction. Application of the simulation algorithm to the hepatic venous system provided the volumetric estimation of the hepatic vein branch drainage area, which is needed for LDLT reconstruction. Using the same simulation software as ours, Yonemura et al. [74] proposed graft selection flow chart for LDLT according to the graft volume and congestion volume (*Fig. 3*).

Figure 3. **Flow chart for the graft selection. Initially the left lobe is considered as a graft. The right lobe is selected if the estimated extended left + caudate lobe volume of the donor is less than 35% of the standard liver volume (SLV) of the recipient. If a remnant liver volume is under 35% of the total liver volume, this donor will be rejected. If congestion volume (CV) is over 25%, or the deducted CV from the graft volume (GV) is under 40% of the recipient SLV, reconstruction of venous tributaries is needed (from [74])**



3D virtual cholangiography

Although biliary variants can be depicted by means of intraoperative cholangiography, this procedure results in time delays and does not permit the surgeon to freely adjust the surgical strategy [75]. Endoscopic retrograde cholangiography represents an invasive technique and is associated with a considerable number of complications (e.g., iatrogenic pancreatitis), thus potentially subjecting the voluntary donors to a higher risk than with CT cholangiography. Standard MR cholangiopancreatography techniques based on T1-weighted MR images have been shown to be insufficient to depict the normal intrahepatic bile ducts beyond the hepatic bifurcation [76]. Yeh et al. [77] performed a comparison of contrast-enhanced CT and MR cholangiography in potential liver donors and confirmed a substantially better visualization of the biliary tract with MDCT. Schroeder et al. [66] reported that contrast-enhanced CT cholangiograms showed the biliary tree at least up to the second, and more often up to third and fourth, intrahepatic branches in 99.6% of all LDLT candidates. The substantial concordance of preoperative and intraoperative biliary anatomical findings was achieved [78]. Our simulation incorporating 3D cholangiography also facilitated preoperative identification of the variant bile duct, of which the recognition was important to avoid donor morbidity [73].

Future studies required

In the future, the impact of these new simulation technologies on surgical education and their exact cost-effectiveness must be assessed objectively [79,80]. Comparative study and even consideration for a randomized trial need taken to document advantage for the new technology over standard practice. It seems likely that future generations of surgeons will be selected, trained, credentialed, and recredentialed using simulation and virtual reality devices.

Conclusions

In conclusion, simulation will both allow training and provide expert knowledge with detailed information useful for the preoperative planning of liver resections. Our novel 3D simulation system, based on the hepatic circulation, provided accurate volumetric and stereotactic information to achieve safe and curative hepatectomy. In addition, our simulation program was applicable to the hepatic venous system to predict liver congestion in LDLT. With increasing use of ablative procedures and laparoscopic surgery, preoperative and intraoperative imaging, and navigation will hold increasing significance for the hepatobiliary pancreatic surgeon.

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Recent concepts in the management of bowel problems after spinal cord injury

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Abstract

Bowel problems after SCI can be debilitating. Colonic inertia as a result of decreased parasympathetic (S2-4) stimulation of the left colon and rectosigmoid seems to be the principal abnormality accounting for DWE. The conventional measures used for decades have poor results in many people. Neostigmine, an anticholinesterase inhibitor, appears to be a more physiological agent for these individuals. The combination of neostigmine + glycopyrrolate infusion has shown encouraging results after intravenous administration and studies are under way to assess the efficacy of neostigmine by other routes.

Introduction

A significant number of individuals with chronic spinal cord injury (chronic SCI) have gastrointestinal (GI) symptoms due to bowel dysfunction [1]. Adequate bowel care is an important part of their management. The intent of this paper is to acquaint physicians with the pathophysiology of bowel problems after SCI and to summarize current concepts in the management of individuals who have sustained such damage.

Magnitude of the problem

According to the most recent data from the National Spinal Cord Injury (NSCI) Database, the prevalence of SCI in the US is approximately 250000 with 12000 new cases each year [2]. About 40-50% of injuries to the spinal cord are due to motor vehicle accidents [3]. The severity of the injury determines the outcome and can be classified using the American Spinal Injury Association (ASIA) impairment scale (*Tab. 1*) into five different stages [4]. The economic burden of this problem is with the direct and indirect (loss of income and productivity) annual cost of managing these individuals estimated to be at least $ 4 billion. These costs are especially high since these injuries typically occur in young males (average age of 37.6 years at the time of injury) [5].

SCI results in permanent disability in about 30-40% of cases [1,6-8]. In addition to the physical limitations due to paralysis, bowel and bladder problems are common. In terms of bladder dysfunction, use of intermittent catheterization has significantly reduced the incidence of urinary tract infections and improved the survival rate [9].

As a result, bowel dysfunction has become a more major issue [1,6-8]. To manage this problem effectively, it is first important to understand normal neuromuscular coordination of the colon and the pathophysiological changes which occur after SCI.

Neuromuscular coordination of the colon

Normal colonic and anorectal function is important for the process of defecation. The internal anal sphincter (IAS), an involuntary sphincter, is the continuation of the inner circular muscle layer of the colon. In contrast, the external anal sphincter (EAS) is made up of striated muscle layer and is under voluntary control [10]. Normal function of the EAS is important in preventing premature expulsion of feces and its integrity is a major factor in maintaining continence.

The colon is richly supplied with both autonomic (parasympathetic and sympathetic) and somatic (sensory and motor) innervation (*Fig. 1*) [11]. These different pathways are integrated by higher centers in the brain and spinal cord. The parasympathetic innervation of the colon is responsible for colonic contractions and motility. The right and proximal transverse colon are innervated through the vagus nerve while the left colon and rectum receive input from spinal segments S2-S4

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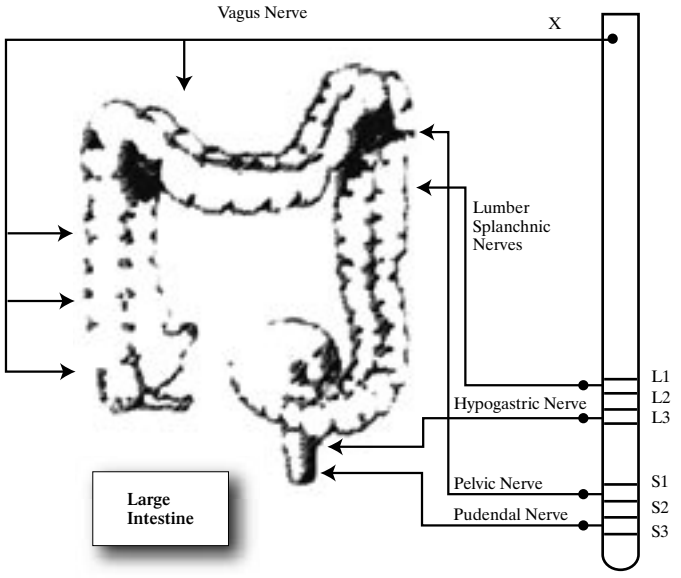


Figure 1. **Extrinsic innervation of the large intestine. The vagus nerve (X) innervates the right colon while propulsive activity in the left colon is mediated by the parasympathetic (pelvic) nerves. Sympathetic innervation (L1-3) via the splanchnic nerves and hypogastric nerves is inhibitory. The anal canal is innervated by voluntary efferent motor fibers to the external anal sphincter via the pudendal nerve from the sacral spinal cord (S2-4)**

Table 1. **American Spinal Injury Association (ASIA) impairment scale**

Grade	Description
A	Complete; no sensory or motor function preserved in the sacral segments S4-S5
B	Incomplete; sensory but not motor function preserved below the neurological level and extending through the sacral segment S4-S5
C	Incomplete; motor function preserved below the neurological level; most key muscles have a grade <3
D	Incomplete; motor function preserved below the neurological level; most key muscle have a grade >3
E	Normal motor and sensory function

Figure 2. **Effect of food ingestion on the motility index (mm Hg). The motility index increased significantly in both SCI ($p<0.01$) and SI ($p<0.02$) subjects after meal ingestion, but to a lesser extent in the latter**

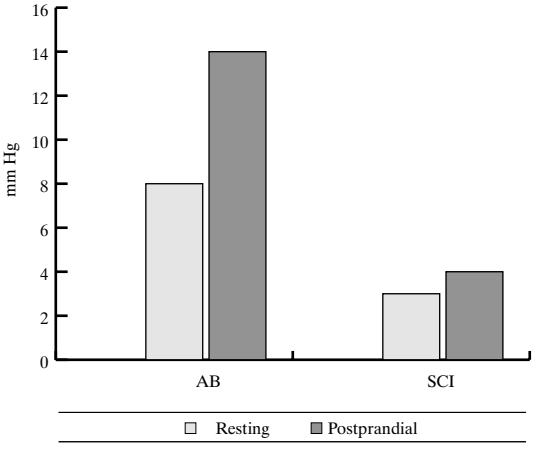
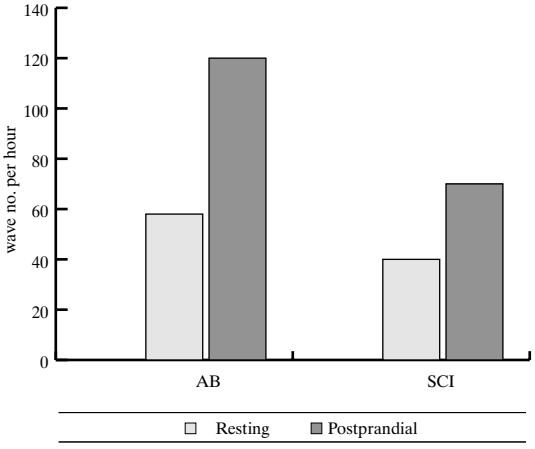


Figure 3. **Effect of food ingestion on the no. of waves per hour. There was a significant increase in the number of waves seen in SCI ($p<0.008$) as well as SI ($p<0.005$) subjects after meal ingestion**



via pelvic nerve or nervi erigentes [11]. The sympathetic supply originates from the lumbar splanchnic nerves and is the major pathway for carrying the sensations from the colon. The somatic fibers innervating the EAS are derived from the pudendal nerve (S2-S4). These nerves directly innervate the colon and also form Auerbach's and Meissner's plexuses within the muscle layers. Together, these plexi constitute what is termed the enteric nervous system (ENS) [10,11].

The neuromuscular innervation of the colon results in both non propulsive contractions under the control of ENS as well as high amplitude propagating contractions (HAPC) [1]. Various neurotransmitters including acetylcholine, catecholamines, and serotonin have been shown to regulate colonic motility. However, the principal autonomic neurotransmitter is acetylcholine [12].

Pathophysiological changes after SCI

Prolonged mouth to cecum transit time (MCTT) has been shown in individuals with quadriplegia using radio-opaque markers [13,14]. Segmental evaluation has also shown pro-

Figure 4. **The effect of food ingestion on the motility index shows regional variation in the SCI group. The increase in motility index (mm Hg) was only significant in the descending colon ($p < 0.03$) and not the rectosigmoid region**

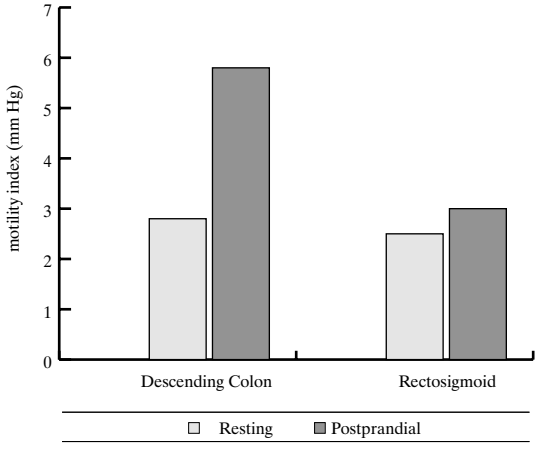


Table 2. **Clinical presentation in patients with SCI due to UMN vs LMN injury**

	UMN lesion	LMN lesion
Level of lesion	Above T10 vertebral or T12 spinal segment	Below T10 vertebral or T12 spinal segment
Transit time (Cecum to anus)	Increased	Increased
Motility of left colon	Decreased	Decreased
EAS	Spastic paralysis	Flaccid paralysis
Sympathetic output	Absent with lesions above T6 spinal segment	Retained
Symptoms	Constipation DWE Incontinence*	Constipation DWE Incontinence
Fecal impaction	Proximal colon	Rectal
Autonomic dysreflexia	Common with injuries above T6 level	Rare
Reflex defecation	Present	Not known

Constipation is <3 bowel movements per week; DWE or difficulty with evacuation is a combination of constipation with bloating, discomfort, pain, and prolonged bowel care sessions; * Patients with SCI due to UMN injury develop incontinence due to loss of sensations and development of lax sphincter later due to use of frequent laxatives and enemas

longed transit time of the left colon in subjects with paraplegia compared to able bodied (AB) individuals [15,16]. Our group has studied colonic motility in different segments of the left colon after SCI (4 subjects with paraplegia and 4 with quadriplegia). The results were compared to findings in 6 matched AB individuals [17]. Motility was evaluated for 1 h before breakfast and for 1 h during meals.

Baseline as well as meal stimulated colonic motility was reduced in SCI subjects compared to AB individuals (*Fig. 2,3*). Regional variations were noted in the SCI group with a post prandial response seen only in the descending colon and not in the rectosigmoid (*Fig. 4*).

We also studied the effect of SCI on colonic contractions in 14 male volunteers (8 with chronic SCI and 6 healthy controls) 1 h before sleep, during the entire period of sleep and 1h after sleep [18]. It was shown that HAPC are absent during sleep in both SCI and control groups. However, arousal from sleep failed to restore HAPC in subjects with SCI [18].

It appears that prolonged colonic transit time and absence of HAPC contributes to constipation and difficulty with evacuation (DWE) after SCI. As colonic motility depends on adequate colonic parasympathetic tone, these results, in part, were consistent with an absolute or relative loss of such autonomic tone.

Bowel problems with chronic SCI

Problems with defecation become more prominent as time progresses after the acute injury [1]. The clinical picture depends on whether the injury is upper motor neuron (UMN) (above vertebral T10 level) or lower motor neuron (LMN) (below vertebral T10 level) as shown in *Tab. 2*. Problems with defecation in both types of injuries have a significant impact on quality of life in individuals with chronic SCI given the prolonged amount of time spent on their bowel care [1,6-8,19].

In addition, complications such as fecal impaction and autonomic dysreflexia can occur. Fecal impaction is the most common problem often presenting with atypical symptoms such as paradoxical diarrhea, abdominal pain, nausea, vomiting, acute confusional states, urinary symptoms, and rectal bleeding due to pressure ulcerations [20]. Autonomic dysreflexia, occurs in patients with SCI above T6 spinal segment. It is due to an autonomic response to stimuli such as fecal impaction, bladder distension, catheterization, digital rectal stimulation, and colonoscopy [21,22]. Common symptoms are pounding headache, sweating, parasthesias, nasal obstruction, and goose flesh. Hypertension is the most common clinical sign and is seen in 90% of these cases [21]. Although rare, potentially fatal complications of autonomic dysreflexia include seizures and subarachnoid hemorrhage [23].

Management

Effective bowel management in individuals with SCI is of utmost importance. An adequate bowel regimen depends on many factors and will vary from patient to patient, but achieving effective evacuation and preventing incontinence is the common goal [24]. It is, therefore, important to completely evaluate the patient before designing a bowel regimen for any patient with a SCI.

Table 3. **Conventional management strategies for bowel symptoms in SCI individuals**

1.	**Dietary changes**
	a) Fiber diet
	b) High fluid intake
	c) Avoid foodstuffs which cause problems
2.	**Positioning during bowel care**
	a) Toilet seat/commode chiars
	b) Left lateral position for bowel care in bed
3.	**Stimulation**
	a) Digital stimulation of rectum
	b) Abdominal belt
4.	**Fiber**
	a) Soluble (pectin, guar, ispaghula, etc.)
	b) Insoluble (cellulose, legnin, etc.)
5.	**Laxatives**
	a) Bulk laxatives (docusate sodium, potassium)
	b) Stimulant laxatives (senna, bisacodyl, castor oil, etc.)
	c) Saline laxatives (magnesium hydroxide, sodium citrate, sodium biphosphate)
	d) Hyperosmolar laxatives (lactulose, sorbitol, polyethylene glycol)
6.	**Suppositories**
	a) Vegetable oil based bisacodyl suppository
	b) PEG based bisacodyl suppositor
	c) CO2 suppository
7.	**Enemas**
	a) Plain water enemas
	b) Fleet enema (sodium biphosphate)
	c) Therevac (TVC) mini enemas
8.	**Prokinetic drugs**
	a) Metoclopramide for short term use
	b) Cisapride not available for routine use
	c) Other agents like tegaserod require further evaluation
9.	**Surgical options**
	a) Sacral posterior rhizotomy
	b) Sacral anterior nerve root stimulation
	c) Appendicostomy and antegrade continent enema of Malone (MACE)
	d) Colostomy

History

There should be particular emphasis on duration and level of injury, bowel habits before the SCI and pre-SCI dietary habits (fluids, fiber, meal frequency, spices, amount). Medications with potential effects on bowel function should be ascertained and the social support system of the individual should be evaluated.

Physical examination

Patients with SCI may not report symptoms [1,6-8,19]. Particular emphasis should be placed on the person's nutritional and hydration status, the abdominal examination (distension, bowel sounds, tenderness, rigidity, fecal impaction, organomegaly), the rectal examination (hemorrhoids, sphincter tone, impaction, masses, stool guiac), and the neurological examination (level and nature of injury).

Laboratory evaluation

Laboratory evaluation should include a complete blood count, electrolytes, renal and liver function tests, amylase, and plain x-ray of abdomen.

Conventional measures for bowel care

Effective bowel care for individuals with SCI usually involves a number of different strategies (*Tab. 3*). Depending on the social needs and the bowel habits of the individual, frequency of bowel care can be tailored to each individual. Whenever possible, bowel care should be performed in either a normal position or the left lateral position [25]. Digital rectal stimulation (DRS) can also be useful [26]. In our own evaluation of 6 subjects with SCI (4 paraplegics and 2 with quadriplegia), use of DRS was shown to increase both the amplitude and frequency of colonic contractions of the left colon [27]. This anocolonic reflex probably involves stretch receptors in the IAS which increase the parasympathetic output to the left colon. All these patients had SCI of UMN type and whether a similar reflex is present in those with SCI of the LMN type is not known [27]. Diet has an important place in these individuals and minor changes in the diet can help these individuals tremendously. It is important for these individuals to consume adequate amounts of fiber and drink at least 2-3 liters of fluids every day [28]. Supplemental fiber may be needed if dietary intake is inadequate (<30 g/d). Fiber produces uniform stool consistency by absorbing excess water [29,30].

Laxatives are often employed as an adjunct to routine bowel care (*Tab. 3*). Bulk laxatives such as docusate and osmotic laxatives such as lactulose are the most commonly employed preparations [31,32]. Enemas are not promoted for routine use unless needed for fecal impaction.

These conventional strategies are time consuming and expensive and do not target the basic pathology of decreased colonic motility. Perhaps as a result, routine bowel care regimens do not yield satisfactory results in many patients. Hence, there is a need of more effective agents which attempt to reverse the basic pathophysiology after SCI.

Newer modalities

Cisapride, a prokinetic drug acts by increasing the release of acetylcholine from post-ganglionic nerve endings. Studies have documented a reduction in mouth-anus transit time and mouth to cecum transit time in subjects with quadriplegia using this drug [13,33]. We have shown the effect of cisapride in improving MCTT in subjects with SCI [13]. Though generally safe, cisapride has been linked to serious cardiac arrythmias (torsades de pointes) and has been withdrawn from the market [34].

Neostigmine, an inhibitor of enzyme acetylcholinesterase, results in increased levels of acetylcholine at the nerve endings and increases colonic peristalsis. It has been used successfully in patients with acute intestinal pseudo-obstruction [35]. Unfortunately, neostigmine also increases airway resistance and causes bradycardia in a significant number of patients. However, we have shown that these unwanted side effects can be prevented if neostigmine is administered together with glycopyrrolate. The latter is an anticholinergic which appears to spare the muscarinic receptors of the colon [17]. Recently, we have shown

***Figure 5*. Semi-quantitative measure (score of 0 to 4) of bowel emptying using barium oat-meal paste. Evacuation scores: a=1, b=2, c=3, and d=4**

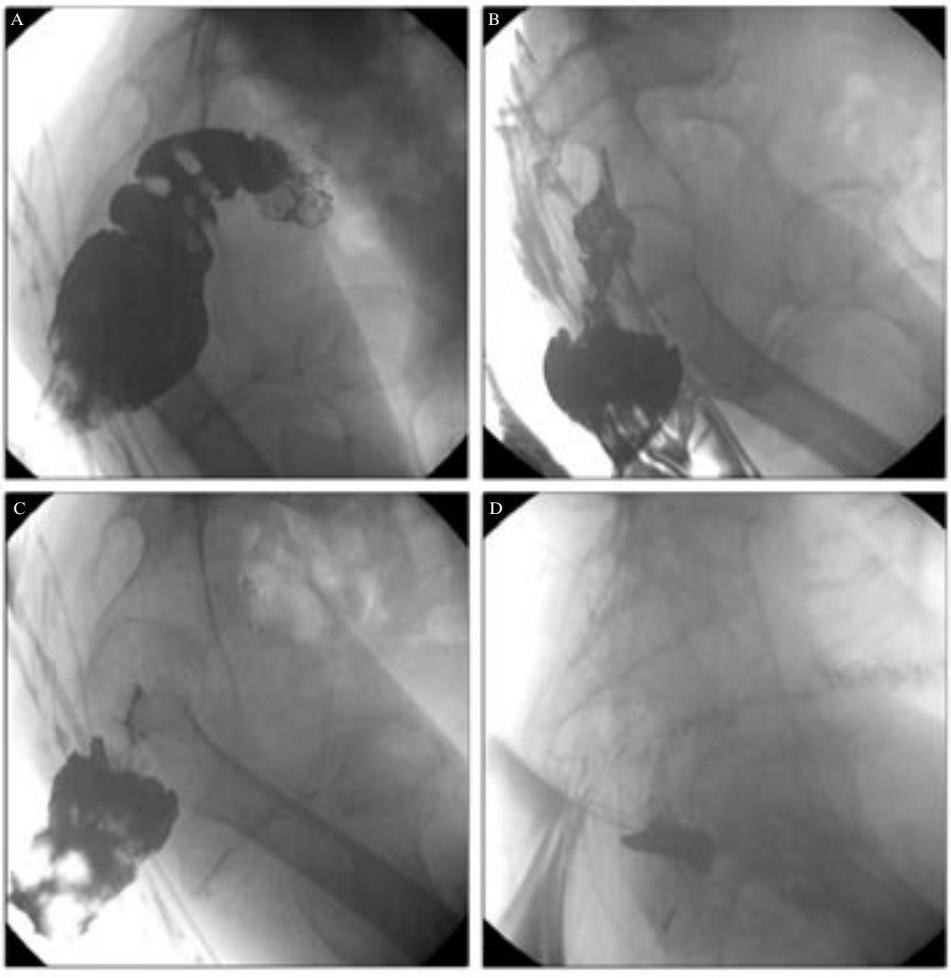


***Figure 6*. Histogram showing the effect of normal saline (control), IV neostigmine (2 mg), and IV neostigmine (2 mg) + glycopyrrolate (0.4 mg) on evacuation of oat-meal barium paste from the rectum and descending colon. The evacuation score was 3 or more in most subjects receiving neostigmine (57%) or combination of neostigmine and glycopyrrolate (64%). None of the subjects scored 2 or more after normal saline**

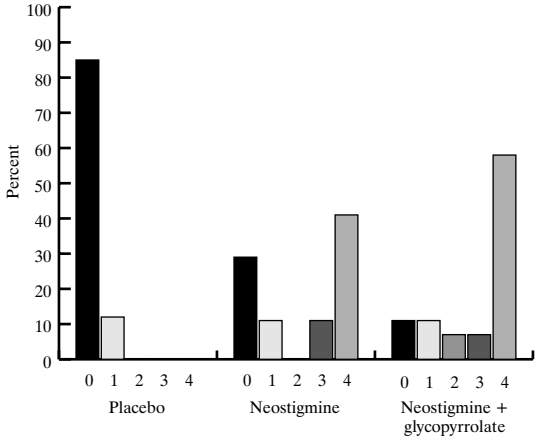
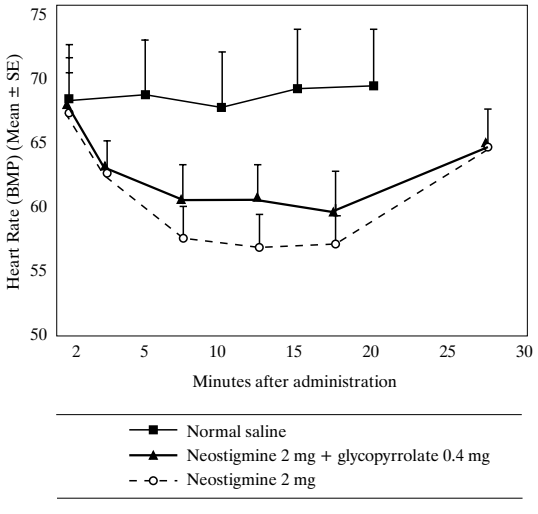


***Figure 7*. Comparison of the effect of normal saline, neostigmine, and neostigmine + glycopyrrolate on the mean heart rate at 5 min intervals**



beneficial effects of neostigmine on the gastrointestinal tract in individuals with SCI [36]. Thirteen individuals with SCI (5 with quadriplegia and 8 with paraplegia) were infused normal saline, neostigmine 2 mg, or neostigmine 2 mg with glycopyrrolate 0.4 mg on separate days. Bowel evacuation was measured by videofluoroscopy after rectal instillation of 200 ml of oat-meal paste of barium (having the consistency of soft stool). Evacuation was measured by an X-ray taken after 30 min and compared with a baseline X-ray (*Fig. 5*). In addition, airway resistance and hemodynamic parameters (pulse and blood pressure) were assessed. Both neostigmine and the combination of neostigmine with glycopyrrolate resulted in better evacuation compared with normal saline (*Fig. 6*) [36].

Although both neostigmine alone and neostigmine with glycopyrrolate resulted in bradycardia, lowest heart rates were recorded when neostigmine was given alone (*Fig. 7*). Both total and central resistance increased with neostigmine relative to normal saline, whereas, neostigmine with glycopyrrolate reversed this (+27% and +17% vs –10% and –8% respectively). The drug was well tolerated except for mild and transient (<30 min) muscle twitching (92%) and abdominal cramps (in those with injury below T10). Although intravenous infusion is not practical for routine clinical use, it remains to be established whether other routes of administration such as subcutaneous or intramuscular are effective in management of these individuals. These trials are ongoing and appear to be encouraging [37].

Tegaserod, a 5HT-4 (serotonin) receptor agonist is another agent with a potential for managing bowel symptoms in SCI. Serotonin has been documented as one of the neurotransmitters implicated in colonic motility [12,38]. Tegaserod in experimental studies has demonstrated an increase in both small bowel and colonic transit [39,40]. The drug has been successfully used in individuals with IBS, pseudo-obstruction, and habitual constipation [41-43]. There are no available data of its use in SCI and this is an area which needs to be explored.

Beneficial effect on the colonic motility of another 5-HT4 agonist, mosapride has been shown in a guinea pig model of SCI (after destruction of L1-3 and S2-4 cords) [44]. In response to rectal distension with a rectal balloon instilled with water, rectal pressures (R-R reflex) and internal anal sphincter relaxation (R-IAS reflex) were recorded at baseline and after intravenous administration of mosapride. Reflex area was derived and expressed as positive values for rectal contractions and IAS relaxations. Reflex indexes (R-R and R-IAS) were calculated as relative ratio of the reflex areas at baseline (control) and after drug administration. The authors showed that mosapride, given intravenously, increased the R-R and R-IAS indexes in a dose dependent manner. These changes could be reversed by about 50% after intravenous administration of the 5-HT4 antagonist GR-113808 [44].

Colostomy is an option in patients with severe and intractable problems [45,46]. It is also frequently advocated as an adjunct in the treatment of perineal pressure ulcers. Stone, et al. [46] showed that objective testing of the transit time can help in deciding the site of colostomy. A sigmoid colostomy is an option for those with normal colonic transit time and inability to adequately evacuate rectum. In contrast, a right transverse colostomy is useful for those with prolonged left colonic transit time. An ileostomy is generally reserved for individuals with a dilated, non-functional right colon. Stone, et al. [46], using a questionnaire, showed that colostomy simplified bowel care, relieved abdominal distension, and prevented fecal incontinence. The time spent in bowel care also decreased significantly from 98.6 min/day before colostomy to 17.8 min/day after colostomy. These individuals represent a high risk for abdominal surgery and selection of the patient is, therefore, important. In a small series, Deshmukh, et al. [47] reported a 15% mortality after colostomy in individuals with large pressure ulcers.

Moreover, Stone, et al. [46] noted postoperative complications in 10% individuals who underwent this procedure. All 27 patients in the first report had a colostomy performed for pressure ulcers whereas in the later, 13 out of 20 patients had colostomy for chronic intractable GI problems (one for rectal cancer), only 7 of 20 had this procedure for pressure ulcers. The authors in the later study performed colonic transit time and anorectal manometry in 6 patients in order to select the colostomy site. These differences could possibly explain the difference in mortality in the two reports. On the whole, it is an acceptable procedure provided it is done in a properly selected person at an appropriate time [45-47].

Surgical posterior rhizotomy and sacral anterior root stimulation are other surgical options shown to have therapeutic utility in SCI patients [48,49]. However, the high overall costs of these procedures has limited their utility. Cutaneous appendicoecostomy has been used to treat intractable incontinence in these patients.

Initially used by Malone, the technique (Malone Antegrade Colonic Enema or MACE) involves administration of enemas through the opening when required [50].

The technique has been shown to be successful in 57% of SCI patients with significant improvement in their QOL [51]. Bowel cleansing can also be accomplished in retrograde fashion using 'pulsed irrigation evacuation' (PIE) [52].

However, its efficacy remains to be determined in a controlled clinical trial.

Management of GI complications

The presenting symptoms of acute abdomen in SCI are quite variable given the sensory loss that accompanies SCI. Therefore, non-specific symptoms such as abdominal distension, vomiting, constipation always require a thorough evaluation.

An accurate diagnosis requires a careful clinical examination, laboratory evaluation, and expedited imaging studies (plain abdominal X-ray and CT scan of the abdomen).

Autonomic dysreflexia (AD) Prevention is the first step in treatment. Once recognized, however, AD should be treated as a medical emergency. If possible the stimulus should be identified and immediately removed. If needed, nifedipine and topical nitrates can be used for emergency control of the blood pressure [21].

Fecal impaction Rectal examination should always be performed if fecal impaction is suspected. If the rectum is empty, imaging is required to assess for more proximal impaction or signs of obstruction. To avoid complications, impaction

should be addressed quickly; delaying treatment for more than 3 days can be hazardous [20]. When an impaction exists, manual evacuation is the first option and requires proper lubrication and local anesthesia. When the impaction is beyond the reach of finger, sigmoidoscopic lavage can be effective. In addition, gastrograffin and golytely have been effective [53]. If these procedures fail, surgery is a last resort.

Colorectal cancer (CRC) screening

Individuals with SCI are at risk of acquiring the same degenerative conditions including cancer, as able bodied people. In a population based study in veterans, the incidence of CRC in patients with SCI similar to that in the general population [54].

The anatomic distribution of CRC was also the same as in the general population with two third of the lesions occurring on the left side or the rectum [54]. However, in contrast to able bodied population, 60% of these tumors were found to be quite advanced (stage III or IV) at the time of presentation. The inability to differentiate symptoms of colorectal carcinoma from other GI complaints in individuals with SCI probably accounts for the delay in diagnosis of colorectal cancer [54]. Even more than in able bodied individuals, early detection and cure of CRC requires regular colonoscopy as a routine measure.

Colonoscopy in individuals with SCI has unique features. Not only must the preparation of the colon be adapted to SCI, but the performance of the procedure must be modified. In this respect, we have found that a two day preparation with oral phosphosoda and golytely is often required. Moreover, we have noted that SCI patients have difficulty in retaining the insufflated air, have lower cecal intubation rate (82%) and have relatively poor colon preparation.

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Current topics on precursors to pancreatic cancer

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Abstract

Prognosis of invasive pancreatic ductal adenocarcinoma is bleak and the vast majority of patients with pancreatic cancer die of their disease. The detection and treatment of the non-invasive precursor lesions of pancreatic cancer offer the opportunity to cure this devastating disease and therefore great efforts are being made to identify the precursors to pancreatic cancer. Several distinct precursor lesions have been identified. Mucinous cystic neoplasms (MCNs), intraductal papillary mucinous neoplasms (IPMNs), and pancreatic intraepithelial neoplasias (PanINs) all harbor varying degrees of dysplasia and stepwise accumulation of genetic alterations, suggesting progression of these lesions from benign toward malignant neoplasms. MCNs have a characteristic ovarian-type stroma. About one-third of MCNs are associated with invasive carcinoma of ductal phenotype. IPMNs are recently established clinical entity with characteristic features of mucin hypersecretion and duct dilatation. Some IPMNs are associated with invasive carcinoma and IPMNs are recognized precursors to pancreatic cancer. PanINs are microscopic proliferative lesions arising from any parts of the pancreatic duct system. Low grade PanINs are commonly found in pancreatic ducts of elder individuals, while high grade PanINs, previously called carcinoma *in situ*/severe ductal dysplasia, may eventually give rise to invasive pancreatic cancer. Appropriate clinical managements are requisite for patients with MCNs, IPMNs and PanINs. Further investigation of these precursor lesions is expected to reduce the mortality from pancreatic cancer.

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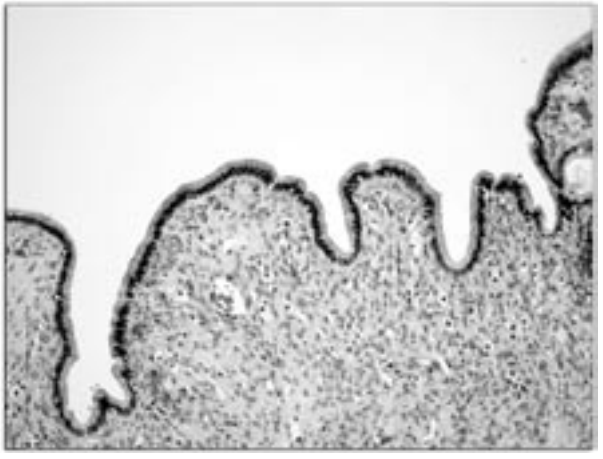
Introduction

Pancreatic cancer affects 213,000 people every year and 98% of the patients die from their disease despite of numerous therapeutic attempts utilizing most intensive multi-modality treatments [1]. Hence, early detection and early treatment have been thought to be the best approach to reduce mortality from pancreatic cancer [2]. However, even small, <1 cm, invasive pancreatic cancers prove fatal [3]. Accordingly, we need to focus on detecting and treating pre-invasive precursor lesions in the pancreas. Previous clinicopathological and molecular studies have revealed three important pre-invasive precursors to pancreatic cancer; mucinous cystic neoplasms (MCNs), intraductal papillary mucinous neoplasms (IPMNs), and pancreatic intraepithelial neoplasias (PanINs) [2]. In this review, we describe the current topics on MCNs, IPMNs, and PanINs with special reference to their pathologic features, molecular genetics, and clinical managements.

Mucinous Cystic Neoplasms (MCNs)

Mucinous cystic neoplasm (MCN) is a cystic neoplasm composed of mucin-producing epithelial cells and densely packed spindle-shaped cells beneath the epithelium. The band of stromal cells beneath the neoplastic epithelium is called "ovarian-type" stroma, an important diagnostic criteria of MCNs (*Fig. 1*). MCNs are found more prevalently in the body-tail than in the head of the pancreas, almost exclusively in female patients with the average age of 40 to 50 years at diagnosis [4,5]. Most MCNs do not communicate with the pancreatic ducts. The dysplasia in the neoplastic epithelium of MCNs can range from minimal dysplasia with uniform nuclei (MCN with low-grade

Figure 1. **Ovarian type stroma in a mucinous cystic neoplasm. Spindle-shaped cells with fibroblastic differentiation including abundant rough endoplasmic reticulum and surrounding collagen compose a band of ovarian type stroma beneath the neoplastic epithelium**



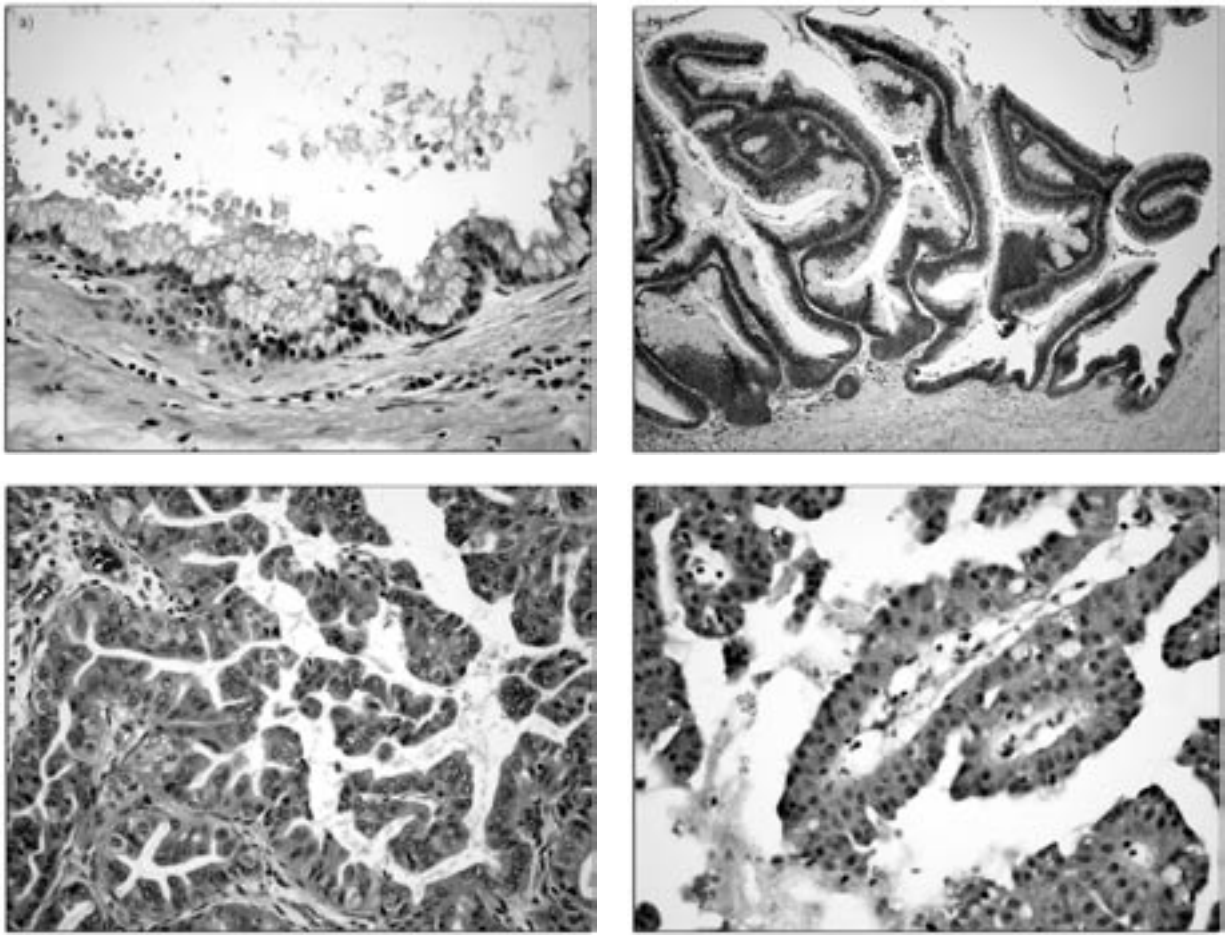
dysplasia or mucinous cystic adenoma), to moderate cytologic and architectural atypia (MCN with moderate dysplasia), to significant architectural and cytologic atypia (MCN with high-grade dysplasia or mucinous cystic carcinoma) [4]. About one-third of MCNs have an associated invasive carcinoma reportedly [4,5]. The invasive carcinoma associated with MCNs is usually tubular adenocarcinoma of ductal phenotype, but examples of undifferentiated carcinomas with osteoclast-like giant cells and sarcomatoid carcinoma have also been reported [5-7]. Clinicopathological features of MCNs indicate that MCN with mild dysplasia can progress to MCN with moderate dysplasia, to MCN with carcinoma *in situ*, and eventually to invasive carcinoma [8-10]. Molecular and immunohistochemical studies have demonstrated that the progression from MCNs with low-grade dysplasia toward MCNs with invasive carcinoma is associated with the accumulation of genetic alterations in *KRAS*, a protooncogene, and tumor suppressor genes including *TP53* and *SMAD4*/*DPC4* [11-14]. The *KRAS* oncogene is located on chromosome 12p and activated by point mutation mostly at codon 12 in approximately 90% of pancreatic cancers [15]. The Ras protein produced by wild type *KRAS* binds to GTPase-activating protein (GAP) and regulates cell cycle progression via the mitogen-activated protein kinase (MAPK) and AKT cascades [16]. Activating mutations, such as those observed in MCNs, impair the intrinsic GTPase activity of the *KRAS* gene product, resulting in a protein that is constitutively active in intracellular signal transduction. *KRAS* mutations were detected in 20% of benign (mild dysplasia), 33% of borderline (moderate dysplasia), and 89% of malignant MCNs (marked dysplasia/carcinoma *in situ* and invasive carcinoma) [12]. The *TP53* gene on chromosome 17p is inactivated in approximately 50-75% of pancreatic adenocarcinoma mostly by a combination of intragenic mutation and loss of the second wild-type allele [17]. Inactivation of *TP53* results in abrogation of p53 protein function and nuclear accumulation of abnormal p53 protein. Abrogation of p53 protein function permits the cells to bypass DNA damage checkpoints and apoptotic signals [18] and may contribute to genomic instability observed in pancreatic cancers [19]. Immunohistochemical studies showed overexpression of the p53 protein in MCNs with the high-grade dysplasia but not in MCNs with mild dysplasia [11,12,14]. *SMAD4* or *DPC4* (*D*eleted in *P*ancreatic *C*arcinoma *4*) is a tumor suppressor gene on chromosome 18q21 identified in a frequently deleted region in pancreatic adenocacrcinoma. Loss of Smad4/Dpc4 protein function interferes with intracellular signaling cascades downstream of the transforming growth factor β (TGF-β) receptors, leading to decreased growth inhibition and uncontrolled proliferation. Immunolabeling for Smad4/Dpc4 protein expression, a specific and sensitive marker of *SMAD4*/*DPC4* gene status, demonstrated loss of expression in the majority of invasive carcinomas associated with MCNs [13].

Case reports have documented patients with incompletely resected mucinous cystic neoplasms with low-grade dysplasia who subsequently developed an invasive adenocarcinoma [8,9,20]. Invasive carcinomas may arise very focally in an MCN [4]. Therefore, when a diagnosis of MCN is established, the lesion should be completely resected, and further, the resection specimen should be thoroughly histologically sectioned to exclude a focally invasive component. Distal pancreatectomy with splenectomy is indicated for mucinous cystic neoplasms with or without invasion in the body and tail of the pancreas. Spleen-preserving distal pancreatectomy either by open or laparoscopic approach may be indicated for non-invasive MCNs depending on the expertise of surgeons and institutions. Once a complete resection is carried out for a non-invasive MCN, only minimal follow-up is required because multifocal MCNs are extremely rare. On the other hand, local recurrence and liver metastasis may occur after resection of deeply-invasive adenocarcinomas arising in association with a MCN [20].

Intraductal Papillary Mucinous Neoplasms (IPMNs)

Intraductal papillary mucinous neoplasm (IPMN) is a grossly visible, non-invasive mucin producing, predominantly papillary or rarely flat epithelial neoplasm arising from the main pancreatic duct or branch ducts, with varying degrees of ductal dilatation [21,22]. IPMNs include a variety of cell types with a spectrum of cytologic and architectural atypia and are classified into IPMN with low-grade dysplasia (intraductal papillary mucinous adenoma), IPMN with moderate dysplasia, and IPMN with high-grade dysplasia (intraductal papillary mucinous carcinoma) [4]. Approximately one-third of IPMNs are associated with invasive adenocarcinoma [4,23]. The invasive carcinoma associated with IPMNs show either a colloid pattern (mucinous noncystic carcinoma) or a tubular pattern (conventional ductal adenocarcinoma) of growth [4,23-25]. The prognosis for patients with an IPMN with an associated invasive colloid carcinoma is significantly better than is the prognosis for patients with an IPMN with an associated infiltrating ductal (tubular) adenocarcinoma [24,26]. As was true for MCNs, clinicopathological finding indicate that non-invasive IPMNs can progress from IPMN with mild dysplasia,

***Figure 2*. Typical histological images of subtypes of intraductal papillary mucinous neoplasms. A, the gastric type; B, the intestinal type; C, the pancreatobiliary type; D, the oncocytic type (intraductal oncocytic papillary neoplasm)**



to IPMN with moderate dysplasia, to IPMN-carcinoma *in situ*, and eventually to invasive carcinoma [27]. This is supported by evidence at the molecular level as well. Analyses of the patterns of loss of heterozygosity in microdissected IPMNs are consistent with clonal progression in IPMNs [4,28,29]. The frequency of *KRAS* mutations increases progressively in accordance with the grades of dysplasia in IPMNs [30,31]. IPMNs with high-grade dysplasia often show inactivation of *TP53*, while *TP53* remains intact in IPMNs with low-grade dysplasia [32-35]. Interestingly, expression of *SMAD4*/*DPC4* is almost always retained in IPMNs regardless the grade of dysplasia.

IPMNs exhibit a variety of architectural patterns with distinct differential features [36,37]. An international collaborative study by Furukawa et al. have suggested that IPMNs can be subdivided into four subtypes; the gastric type, intestinal type, pancreatobiliary type, and oncocytic type, based on the direction of differentiation and mucin expression profile of the epithelium [38] (*Fig. 2*). Gastric-type IPMNs have a slightly eosinophilic cytoplasm, basally oriented nuclei, and abundant apical cytoplasmic mucin [4,37-39]. The gastric-type is negative for MUC1 and MUC2 immunostaining and is also called "null type". Intestinal type IPMNs resemble villous adenomas of the large intestine forming long villous projections lined by columnar mucin-producing neoplastic cells with cigar-shaped nuclei. The neoplastic cells of intestinal type express MUC2 and CDX2, a transcription factor and determinant of intestinal differentiation [4,14,26,38-41]. The pancreatobiliary type of IPMNs is composed of a cuboidal epithelium that forms more complex papillae with bridging and cribriforming [4,26,38]. The epithelial cells of pancreatobiliary type are usually associated with high-grade dysplasia and immunolabeled for MUC1, while they do not express MUC2 [14,38]. The oncocytic type is a rare variant of IPMNs, which is also called intraductal oncocytic papillary neoplasm (IOPN). IOPNs are characterized by abundant eosinophilic cytoplasm, large round nuclei, and prominent and the epithelial cells are associated with high-grade dysplasia and often immunolabeled for MUC1 [38,42]. Mucin expression profile as well as morphological analysis suggests that the intestinal-type IPMNs may progress toward mucinous noncystic (colloid) type of invasive carcinoma, which is negative for MUC1 but express MUC2. The pancreatobiliary type IPMNs may progress toward invasive tubular adenocarcinoma, which is typically MUC1 positive and MUC2 negative [40]. Thus, the subtypes of IPMNs may have a significant clinical implication. However,

it should be noted that there are considerable overlaps among the subgroups, and that a single IPMN can contain more than one type of epithelium. The frequent coexistences of multiple subtypes of IPMNs suggest that one subtype may transform into another subtype, e.g., from gastric type to intestinal type and pancreatobiliary type, or from intestinal type to pancreatobiliary type.

As is true for MCNs, non-invasive IPMN can be cured by complete resection of the lesion, while IPMN with an associated invasive carcinoma may relapse after resection and result in a poor prognosis [26,43]. Ideally, IPMNs should be resected before non-invasive IPMNs progress toward invasive carcinoma. With increasing use of imaging technology including abdominal computed tomography (CT), ultrasound, and magnetic resonance imaging (MRI), there are more and more chances to detect non-invasive IPMNs [44-46]. It is true that some non-invasive IPMNs remain indolent possibly for a long span and, with the trends of augmented chances to detect these lesions, it became of great importance to select IPMNs with significant risks to progress toward potentially lethal invasive carcinoma for immediate curative surgery. On the other hand, some small non-invasive IPMNs may be followed clinically without surgical intervention because they can behave indolently for the lifetime of patients. In order to address appropriate medical care for patients with IPMNs, Tanaka et al. have developed international guidelines for the management of IPMNs [47]. The majority of IPMNs involving the main duct (main duct type) are malignant (carcinoma *in situ* or invasive) and the international guidelines recommended that all main duct type IPMNs should be surgically resected as long as the patient is a good surgical candidate with a reasonable life expectancy. By contrast, IPMNs confined to the branch ducts (branch type) often do not significantly change in size [27] and they are less likely to develop invasive carcinoma as compared to the main duct type [25,48-50]. Although the management of patients with branch type IPMNs is controversal, it is generally agreed that branch duct IPMNs less than 1 cm can be followed with annual MRI or thin slice CT [47]. Branch-duct IPMNs 1 to 3 cm in size should be evaluated by endoscopic ultrasound (EUS) and magnetic resonance cholangiopancreatography (MRCP) or endoscopic retrograde cholangiopancreatography (ERCP). Tanaka et al. recommend surgical resection if the main duct is dilated >6 mm and/or if mural nodules are present (47). The frequency of follow-up for non-resected branch duct IPMNs depends on the size of the lesion. Yearly follow-up is recommended if the lesion is <10 mm in size, 6-12 monthly follow-up for lesions between 10 and 20 mm, and 3-6 monthly follow-up for lesions >20 mm [47]. Yamaguchi et al. performed periodic ultrasound study in 81 patients with IPMNs for longer than 3 years and reported that maximum increases of the MPD diameter by 2.2 mm/year, the cyst diameter by 11.3 mm/year, and emergence or increase of the height of the protruding lesion by 3.3 mm/year were predominantly observed in patients with malignant IPMNs with accuracy greater than 80% [51].

Total pancreatectomy, pylorus-preserving pancreaticoduodenectomy, duodenum-preserving pancreatic head resection, middle pancreatectomy, distal pancreatectomy with or without spleen preservation, and other partial pancreatectomies [52] are indicated in patients with IPMNs depending on the location and extent of the lesion(s). Laparoscopic pancreatic resections have been indicated for IPMNs in some specialized centers [53]. The frozen sections of pancreatic cut margin should be carefully evaluated intraoperatively to avoid incomplete resection of IPMNs [54]. Indeed, recurrence of IPMNs after partial pancreatectomy is not uncommon [55,56]. Metachronous occurrence of IPMNs is also possible because some IPMNs are multifocal [26,57-60]. Therefore, careful follow-up is needed after resection of IPMNs. It should be noted that patients with an IPMN have an increased risk of extra-pancreatic malignancies, particularly colorectal, gastric and lung cancers [61,62].

Pancreatic Intraepithelial Neoplasias (PanINs)

Non-invasive epithelial lesions have been recognized in the pancreatic ducts for over a century [63], and a plethora of terms have been used to designate these lesions. In 1999, a nomenclature of pancreatic intraepithelial neoplasia, or PanIN was proposed to establish a standardized classification system for morphologic, clinical, and genetic studies [64]. PanIN is a microscopic papillary or flat non-invasive epithelial neoplasm arising in the pancreatic ducts and characterized by columnar to cuboidal cells with varying amounts of mucin and degrees of cytologic and architectural atypia. PanINs are classified into three histologic grades of PanIN-1, PanIN-2, and PanIN-3, depending on the degree of atypia (*Tab. 1*, *Fig. 3*). Briefly, PanIN-1 is a proliferative lesion without nuclear abnormality and subclassified into PanIN-1A, which consists of flattened epithelium and PanIN-1B, which comprises a papillary architecture. PanIN-3 is associated with severe architectural and cytonuclear abnormalities, but invasion through the basement membrane is absent. PanIN-3 lesions were classically referred to as “carcinoma in situ”. PanIN-2 is an intermediate category between PanIN-1 and PanIN-3 and associated with a moderate degree of architectural and cytonuclear abnormality. PanINs harbor progressive accumulation of alterations in tumor suppressor genes including *CDKN2A*/*INK4A*, *TP53* and *SMAD4*/*DPC4*, in addition to oncogenic mutations of *KRAS* (2,64-69) (*Fig. 3*). Mutations in the *KRAS* are one of the earliest genetic abnormalities observed in the development of pancreatic neoplasia and the frequency of *KRAS* mutations increases progressively with the degree of dysplasia [65,66]. The *CDKN2A*/*INK4A* gene on chromosome 9p21 encodes the cell cycle checkpoint protein P16, which binds to the cyclin dependent kinases Cdk4 and Cdk6, thereby inhibiting binding of cyclin D1, resulting in G1-S cell cycle arrest [70]. Loss of P16 function, seen in virtually all of pancreatic cancers, occurs via several different mechanisms, including homozygous deletion, intragenic mutation with loss of the second allele, and epigenetic silencing by promoter methylation [71-73]. Loss of p16 protein expression occurs as early as PanIN-1 [65,67,69], while immunolabeling of nuclear p53, a surrogate marker for *TP53* mutation, and loss of Smad4/Dpc4 protein expression are restricted to high-grade PanINs or PanIN-3 [65,68,69] (*Fig. 3*).

While the original classification system of PanINs focused on the lesions in the smaller ducts, several case reports sug-

Table 1. **Pathological features of pancreatic intraepithelial neoplasia (PanIN) and related lesions**

Normal: The normal ductal and ductular epithelium is a cuboidal to low-columnar epithelium with amphophilic cytoplasm. Mucinous cytoplasm, nuclear crowding, and atypia are not seen.

Squamous (transitional) metaplasia: A process in which the normal cubiodal ductal epithelium is replaced by mature stratified squamous or pseudostratified transitional epithelium without atypia.

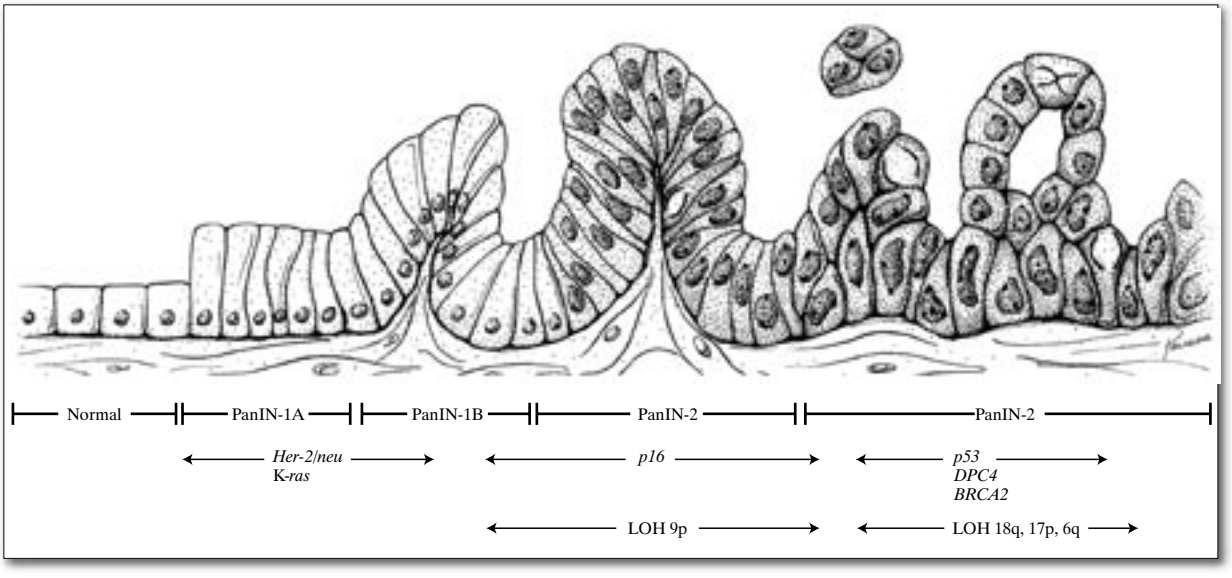
PanIN-1A (pancreatic intraepithelial neoplasia 1-A): These are flat epithelial lesions composed of tall columnar cells with basally located nuclei and abundant supranuclear mucin. The nuclei are small and round to oval in shape. When oval, the nuclei are oriented perpendicular to the basement membrane. It is recognized that there may be considerable histologic overlap between non-neoplastic flat hyperplastic lesions and flat neoplastic lesions without atypia. Therefore, some may choose to designate these entities with the modifier term "lesion" (PanIN/L-1A) to acknowledge that the neoplastic nature of many cases of PanIN-1A has not been unambiguously established.

PanIN-1B (pancreatic intraepithelial neoplasia 1-B): These epithelial lesions have a papillary, micropapillary, or basally pseudostratified architecture but are otherwise identical to PanIN-1A.

PanIN-2 (pancreatic intraepithelial neoplasia 2): Architecturally these mucinous epithelial lesions may be flat but are mostly papillary. Cytologically, by definition, these lesions must have some nuclear abnormalities. These abnormalities may include some loss of polarity, nuclear crowding, enlarged nuclei, pseudostratification, and hyperchromatism. These nuclear abnormalities fall short of those seen in PanIN-3. Mitoses are rare, but when present are nonluminal (not apical) and are not atypical. True cribriform structures with luminal necrosis and marked cytologic abnormalities are generally not seen and, when present, should suggest the diagnosis of PanIN-3.

PanIN-3 (pancreatic intraepithelial neoplasia 3): Architecturally, these lesions are usually papillary or micropapillary; however, they may rarely be flat. True cribriforming, the appearance of "budding off" of small clusters of epithelial cells into the lumen, and luminal necrosis should all suggest the diagnosis of PanIN-3. Cytologically, these lesions are characterized by a loss of nuclear polarity, dystrophic goblet cells (goblet cells with nuclei oriented toward the lumen and mucinous cytoplasm oriented toward the basement membrane), mitoses that my occasionally be abnormal, nuclear irregularities, and prominent (macro) nucleoli. The lesions resemble carcinoma at the cytonuclear level, but invasion through the basement membrane is absent.

Figure 3. **Schematic diagram of pancreatic intraepithelial neoplasias (PanINs) with typical stepwise progression of genetic alterations. The estimated timing of alterations in the K-ras, HER-2/neu, p16, p53, DPC4, and BRCA2 genes is illustrated. Loss of heterozygosity (LOH) in the 9p, 18q, 17p, and 6q chromosomes is also indicated. Artwork by Jennifer Parsons, MA, USA. Reproduced from reference 65 with permission from the publisher**



gested that some PanINs may involve large ducts, including the main duct [74,75]. With recognition of PanINs involving the large ducts, it became necessary to reliably differentiate PanINs involving the main ducts and branch ducts from IPMNs which arise in the same location [76]. To address these issues and further establish international consensus based on clinicopathological and molecular criteria, a meeting of international experts on precursor lesions of pancreatic cancer was held at the Johns Hopkins Hospital in 2003 [22]. In this meeting, guidelines to differentiate PanINs from IPMNs were created (*Tab. 2*).

Accroding to a pathological study in 1174 autopsy patients by Kozuka et al., the incidence of PanIN-1 and PanIN-2 lesions increased progressively with age until they reached 33% and 13%, respectively, at the age over 60 years. PanIN-3 was found in 3% of patients who died in the 7th decade of life, and was twice as frequent in the head as in the body and tail [77]. Most low-grade PanINs remain indolent during the lifetime of an individual, and surgical intervention is not needed for these lesions. In contrast, in several reported cases, PanIN-3 was found at the surgical margin in partial pancreatectomy specimens and

Table 2. **Summary of diagnostic criteria for PanINs and IPMNs**

	PanIN	IPMN
Size	Usually <5 mm	Usually >1 cm
Mucin hypersecretion	No	Yes
Diffuse or cystic dilatation of ducts	Rare	Yes
Tall papillae with stromal core	No	Common
Muc 1 expression	Yes (for high-grade PanINs)	Yes (for PB and oncocytic types) and No (for gastric and intestinal types)
Muc 2 expression	No	Yes (for intestinal type) and No (for gastric, PB and oncocytic types)
Abrogation of SADM4/DPC4	30% in PanIN-3	No
Associated with invasive colloid carcinoma	No	Yes in half of associated invasive carcinoma

invasive carcinoma developed in the pancreatic remnant 17 months to 29 years after the pancreatectomy [78-80]. Hence, it is estimated that some, if not all, PanIN-3 lesions may progress to invasive carcinoma within a span of years and this "window" would provide an unprecedented opportunity to cure pancreatic cancer before invasion. PanIN lesions, especially those arising in the large ducts, can cause segmental narrowing or stenosis of the pancreatic ducts and localized fibrosis of pancreatic parenchyma due to obstruction of the drainage duct [74,75]. On radiologic examination, these findings can indicate an underlying PanIN lesion. However, most PanINs are microscopic lesions and usually too small to be visualized directly by radiological imaging. It is therefore quite unusual that a diagnosis of PanINs is made by conventional imaging techniques alone. The exception occurs in patients from familial pancreatic cancer kindreds, where the entire pancreas is often involved by multicentric PanIN lesions, leading to localized pancreatitis and lobular atrophy occurring in the immediate distal parenchyma. This pattern of "multicentric lobular atrophy" gives rise to a diffuse stippled pattern of pancreatitis discernible on radiologic examination, and is a recently described entity in the setting of familial pancreatic neoplasia [81]. Nevertheless, in patients who do not harbor a familial predisposition, the diagnosis of PanIN lesions is usually made in resection or biopsy specimens under the microscope. Molecular and proteomic analysis of biosamples including pancreatic juice, feces, etc. may be utilized to diagnose PanINs, especially high-grade PanIN lesions, in the future.

Perspectives

The international consensus meeting on precursor lesions of pancreatic cancer held in 2003 has set up a working formula to study MCNs, IPMNs, and PanINs in a systematic manner [22]. The mechanisms involved in the initiation of these precursor lesions and progression toward invasive cancer remain to be elucidated. Further investigations of MCNs, IPMNs, and PanINs from clinical, pathological and molecular aspects are encouraged and we would eventually succeed to reduce the mortality from pancreatic cancer.

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Sperm analyses, genetic counselling and therapy in an infertile carrier of a supernumerary marker chromosome 15

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Abstract

Purpose: A supernumerary marker chromosome (SMC) was analysed after lymphocyte culture of a patient with oligoasthenoteratozoospermia (OAT) before ICSI treatment.

Material and methods: By additional molecular cytogenetic investigations the marker could be identified as a heterochromatic derivate of chromosome 15 [karyotype: 47,XY,+der(15)].

Results: Sperm analyses by interphase FISH showed a normal monosomy 15 in 82% and an additional marker in 17% of the cells. In spite of these findings a pregnancy could not be induced. The brother of the patient showed the same chromosome abnormality and an OAT-syndrome as well.

Conclusions: ICSI-treatment lead to a normal pregnancy and to the birth of a healthy boy. The genetic risk factors of both marker carriers are analysed in detail.

Key words: supernumerary marker chromosome (SMC), derivate of chromosome 15, fluorescence in situ hybridization (FISH), infertile males, oligozoospermia, ICSI-treatment.

Introduction

Chromosome aberrations play an important role in the aetiology of male infertility. Among infertile males, the increase is about 10-fold compared to the general population [1,2]. These abnormalities include numerical changes of the sex chromosomes but frequently balanced sex chromosome and autosome rearrangements also, such as the translocation 13/14 or supernumerary heterochromatic marker chromosomes [3].

Here, we report the case of a patient and his family with a supernumerary marker chromosome (SMC) which was identified as a derivate of chromosome 15. Investigations of somatic cells could be combined with sperm analyses thus allowing a better risk estimation in genetic counseling of the couple.

Material and methods

Clinical and andrological findings

An infertile couple who had wished for children for 10 years, attended the Section of Endocrinology and Reproduction at the Department of Obstetrics and Gynecology of the University of Bonn for further diagnosis and therapy.

Both partners were healthy. Exposition to mutagenic agents could be excluded. The husband was 41 years and his wife 38 years old at the time of examination. The wife had undergone an operative laparascopy 8 years ago, where peritubal adhesions on the left side were removed and minimal endometriosis was diagnosed. Hydropertubation showed that both Fallopian tubes were patent. Apart from a slight luteal insufficiency, all other gynaecological and endocrinological parameters were normal in the woman. The husband was repeatedly diagnosed in the past with mild to moderate oligoasthenoteratozoospermia (OAT). In the first semen analysis at the andrological laboratory of our institution, the sperm count was $12x10^6$/ml, sperm motility was 40% and less than 5% of the spermatozoa showed a normal morphology. The diagnosis was confirmed in a second investigation 9 months later (*Tab. 1*).

Cytogenetics

Conventional chromosome analysis in lymphocytes Chromosome analyses of the couple were performed on metaphase chromosomes from cultured peripheral blood lymphocytes using standard banding techniques (QFQ-, GTG-, RBA-, CBG-banding, NOR and DA/DAPI staining). The woman

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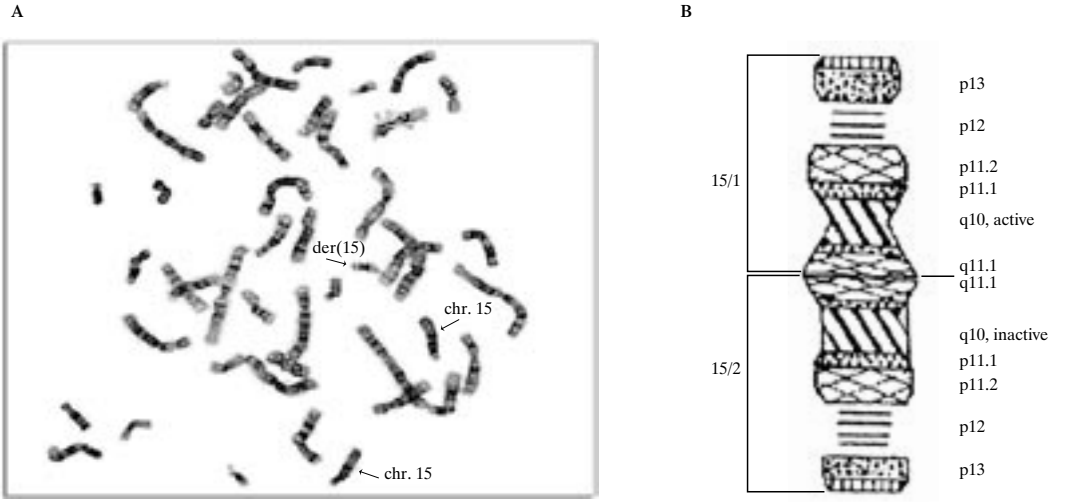
Table 1. **Semen parameters in the patient with oligoasthenoteratozoospermia (OAT)**

Semen parameters	Semen analysis 1	Semen analysis 2	Normal Range (WHO)
Volume	3 ml	4 ml	≥2 ml
Concentration	$12 \cdot 10^6$/ml	$10 \cdot 10^6$/ml	$\geq 20 \cdot 10^6$/ml
Motility	40%	20%	>50%
Normal morphology	<5%	<5%	>30%

Figure 1. **a) Karyotype showing the two normal chromosomes 15 and the der(15): 47,XY,+psu dic(15;15) GTG; b) Ideogram of the SMC(15)**



had a normal female karyotype (46,XX). The husband showed a supernumerary marker chromosome (SMC) in all metaphases (n=50). The marker was bisatellited, dicentric and smaller than chromosome 21 (*Fig. 1*). Analysis of the structure of the short arm regions showed, that the marker was not isodicentric, but originated from chromatid exchanges from two homologous chromosomes 15 (15p11.2, p12, p13 were of different size). The karyotype could be delineated as 47,XY,+der(15).

Family investigation The patient had two siblings, a brother and a sister, both of them healthy. The sister had 3 healthy children whereas in the brother an OAT syndrome was diagnosed as in our patient. Chromosomes were investigated in the siblings and the parents of the patient. While the sister and the father showed normal karyotypes, the brother and the mother had the same supernumerary marker chromosome as the patient. The 38 year-old brother who showed an OAT syndrome received chemotherapy 10 years ago after the diagnosis of a carcinoma testis. No data were available on his chemotherapy. Therefore, today the role of the marker chromosome and the chemotherapy as causes of the OAT syndrome cannot be determined.

The five siblings of the mother could not be examined cytogenetically, but the following peculiarities were noted: one of the brothers had no children in two marriages and one of the sisters none in one marriage. Moreover, one maternal brother was mentally retarded, and one sister died in early infancy.

FISH analyses For further analysis and for exclusion of euchromatic material in the marker chromosome, fluorescence in situ hybridization (FISH) was performed with the commercially available SNRPN/D15Z1/PML probe (Vysis, Downers Grove, IL, USA; *Fig. 2*). The FISH procedure was performed according to manufacturer's instructions. The slides were coun-

Figure 2. **Localisation of the FISH DNA probe set SNRPN/D15Z1/PML**

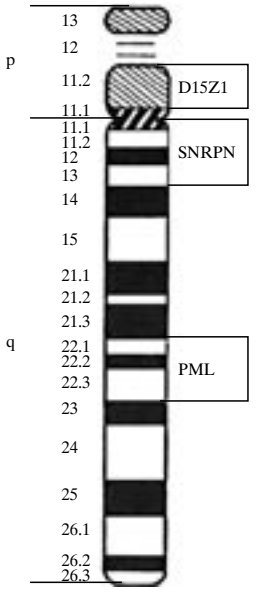


Figure 3. **FISH analysis of lymphocytes with the probe SNRPN/ /D15Z1/PML. Karyotype: 47,XY,+psu dic(15;15).ish psu die(15) (q11.1; q11.1)(D15Z1++,SNRPN-,PML-)**

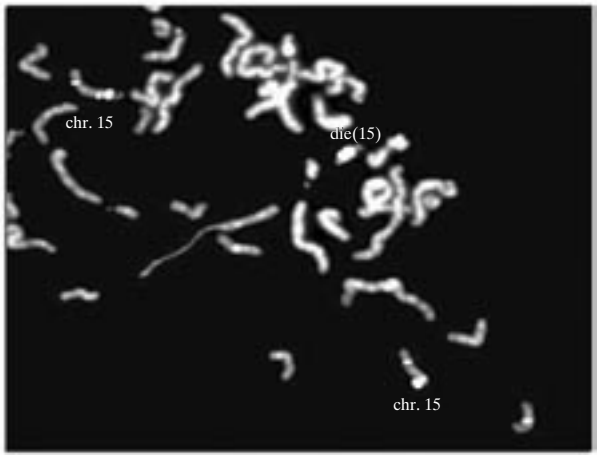
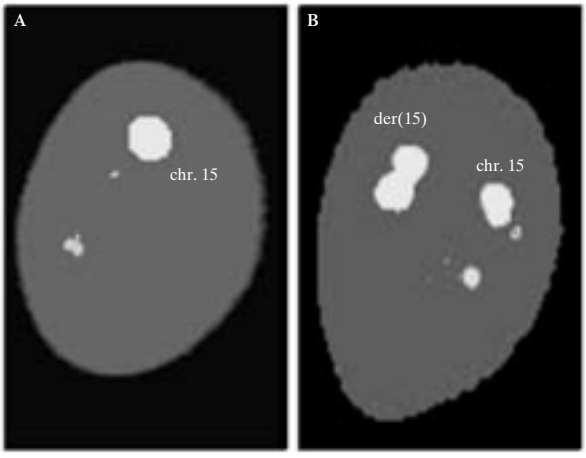


Figure 4. **Sperm nuclei of the patient with der(15) after FISH: A) one chromosome 15; B) one chromosome 15 and one der(15)**



terstained with DAPI/antifade. The probe SNRPN/D15Z1/PML labelled on the 2 normal chromosomes 15 in the three specific regions. The derivate showed two green signals for the D15Z1 region (*Fig. 3*). A euchromatic signal was absent. Thus, the SMC was determined as a heterochromatic dicentric marker derived from chromosome 15 [Karyotype: 47,XY,+psu dic(15;15).ish psu dic(15)(q11.1;q11.1)(D15Z1++,SNRPN-,PML-)].

Sperm preparation and interphase FISH

Methods Semen samples of the consultant and 10 control persons were analysed. The controls were fertile men of a matched age group [4]. The samples were washed three times in phosphate-buffered saline (PBS) and spinned at 1200 rpm for 10 min. The sediments then were fixed in methanol/acetic acid (3:1) and stored at -20°C until further processing. After thawing, the fixed semen samples were washed twice in fresh methanol/acetic acid (3:1), spread on clean glass slides and allowed to dry on air. To render the sperm chromatin accessible to DNA probes, the slides were treated with dithiotreitol (DTT). They were incubated for 3 min in 25 mM DTT/1 M Tris (pH 9.5) at room temperature. After decondensation, they were washed once for five minutes in 2x standard saline citrate solution (SSC), dehydrated through an ethanol series (70-90-100%) and air-dried. Each slide was denatured with 3M NaOH solution at room temperature for 3 min. They were then immersed in a 70, 90, and 100% ethanol series for 3 min each and air-dried. In the semen sample of the patient, interphase FISH was performed with the SNRPN/D15Z1/PML probe (*Fig. 4*) according to the manufacturer's instructions. In the control persons' probes, the FISH was carried out with the CEP 15 probe (Qbiogene).

The slides were examined using a diaplan-fluorescence microscope (LEICA) with the appropriate filter sets: single band pass filter (Aqua, FITC, TRITC) and a triple band pass filter (DAPI/ FITC/TRITC). Only samples with a hybridization rate of >99% were analysed. 5000 sperm nuclei were scored in each control person and 1000 in the patient. Inclusion criteria were the intact spermatozoa with a significant decondensation, and the hybridization signals had to be clear.

Table 2. **Sperm analysis by interphase FISH with probes SNRPN/D15Z1/PML in the patient with additional SMC(15) and in controls with the probe CEP 15**

Signal combinations	Patient (%)	Controls (%)
Monosomy 15	82.70	99.83
Monosomy 15 and der(15)	17.00	-
Disomy 15 and der(15)	0.20	-
Disomy 15	0.10	0.13
Nullisomy 15	0.00	0.04

Statistical analysis The data were analysed with the χ^2-test. The Statistic Package for Social Sciences (SPSS) version 11.0 for Windows was used for statistical calculation. A significant statistical difference was assumed when the P-value was lower than 0.05.

Results

Controls

50000 sperm nuclei from 10 healthy men of the same age group with normal fertility as the patient were analysed by FISH with the CEP 15 α-satellite-DNA probe (Qbiogene). The disomy rate for chromosome 15 was 0.13% and the nullisomy rate was 0.04% (*Tab. 2*).

Patient

1000 sperm nuclei were analysed after hybridization with the chromosome 15 probes SNRPN/D15Z1/PML (*Fig. 3*). In 82.7% of the sperm, there was the combination of three signals for one chromosome 15 per nucleus corresponding to a monosomy 15 (*Fig. 4a*); 17.0% showed one chromosome 15 and the additional marker chromosome (*Fig. 4b*); 0.2% of the nuclei had a disomy 15 and the additional marker, and finally 0.1% showed a disomy

Table 3. **Genetic risk estimation of fetal chromosome syndrome for the consultant with additional marker chromosome 15 and for his wife with increased age at pregnancy**

Maternal:	Increased age of 37/38 years at time of ICSI therapy Risk of aneuploidy 1:100
Paternal:	The SMC has a higher risk of meiotic error. This can lead to: • Uniparental disomy (UPD) 15 following trisomy 15 rescue Phenotype: Angelman syndrome • Trisomy 15 mosaicism due to incomplete trisomy rescue of the aneuploid zygote • Unequal crossing over in paternal meiosis can lead to: – duplication 15 q11.2 => trisomy 15 q11.2 syndrome – deletion 15 q11.2 => Prader-Willi syndrome
	Low risk estimation (less than 1%) for a pathologic development of the embryo due to the marker chromosome
Recommendation:	Prenatal diagnosis

for chromosome 15. Nuclei with a nullisomy for chromosome 15 were not observed (*Tab. 2*).

Comparing the data of our patient with those of the control group, there was a highly significant increase of sperm nuclei with disomy 15 (0.3 vs 0.13%; $p<0.001$), whereas there was no difference for the nullisomy rate between the patient and controls.

In the spermatozoa examined, the additional marker was only observed in combination with one or two normal chromosomes 15.

Genetic counselling and risk estimation

The couple asked for genetic counselling and interpretation of the chromosome findings. Different risk factors had to be taken into account (*Tab. 3*).

The increased maternal age of 38 years bears a higher aneuploidy risk of 1:100 compared to 1:500 in a 25 year-old woman. On the paternal side, the der(15) raises the risk for nondisjunction of chromosome 15. After fertilization, a trisomy rescue mechanism can lead to uniparental disomy (UPD) 15 with imprinting defects or, in the case of incomplete trisomy rescue, to a mosaic trisomy 15. Moreover, unequal crossing over of a normal chromosome 15 with the SMC(15) during male meiosis can result in a duplication or deletion of 15q11.2 in the normal chromosome 15 or in an insertion of euchromatic material in the marker. The duplication is associated with a trisomy 15q11.2 and the deletion with a Prader-Willi syndrome (PWS), but both types of chromosome aberrations are rare events. Despite the combination of maternal (maternal age) and paternal risk factors [SMC(15)], the genetic risk for the offspring in case of a successfully induced pregnancy could be regarded as low (*Tab. 3*).

Infertility treatment

The couple underwent two in vitro fertilization treatments which were combined with intracytoplasmic sperm injection because of reduced sperm quality. In the first ICSI-attempt, two oocytes were retrieved after ovarian hyperstimulation, but due to fertilization failure, no embryos could be transferred. In the second treatment cycle, sixteen oocytes could be obtained and four oocytes were fertilized. Two embryos were transferred to the uterus but no pregnancy could be achieved. The remaining two oocytes were frozen in the two-pronuclear stage, thawed after nine months, cultured and then transferred, again without resulting in a pregnancy.

The patient's brother and his wife were also treated with ICSI. Ten oocytes were obtained after ovarian stimulation, eight of them could be fertilized, six were cryopreserved, and two embryos were transferred without inducing a pregnancy. Three oocytes in the two-pronuclear stage were then thawed and transferred, but again failed to induce a pregnancy. The second transfer with three cryopreserved two-pronuclear stages resulted in an implantation, and a healthy boy was born after an uneventful pregnancy.

Discussion

Supernumerary marker chromosomes (SMC) show a frequency of 1:1 000 in unselected populations. About 50% of them are heterochromatic and thus harmless for all carriers. Derivates of chromosome 15 are the most frequent marker chromosomes with about 40% of all SMC. About 50% of the heterochromatic markers are familial in origin [5,6]. The inheritance occurs preferentially in females. In males the either get lost in the majority of germ cells or lead to infertility as in the family presented here. Several studies reported a higher incidence of SMC(15) in infertile males with oligo- or azoospermia. In contrast, females usually show a normal fertility, which is the reason for the prevalence of maternal inheritance of the familial SMC [7-9].

The patient described here is a carrier of a familial heterochromatic der(15), which is associated with an OAT-syndrome and infertility. We performed dual-colour FISH analysis of sperm to determine the segregation ratio of the SMC(15) during spermatogenesis. Theoretically, there should be a 1:2 ratio for the SMC, but only 17.2% of 1 000 sperm nuclei analysed carried the SMC(15). This segregation ratio of almost 1:5 reflects a selection process against the marker during sperm development. Spermatogenetic failure occurs during prophase of meiosis I, where the marker chromosome preferentially associates with the XY-bivalent, leading to an early disruption of spermatogenesis [10]. This partial spermatogenetic arrest may be the reason for the reduced fertility found in our patient.

The lack of larger study data on segregation ratios of SMC during male meiosis does not allow the generalization of our observations of meiotic inheritance.

The difference in the success of the ICSI treatment in the 2 brothers can be caused by different transmission factors.

First, the age of the wife of our index patient was significantly increased compared to his sister in law.

Secondly, the genetic background in the 2 couples is different and may thus lead to differences in the success of the ICSI-therapy.

Conclusions

A familial supernumerary marker chromosome in 2 brothers with OAT syndrome and maternal inheritance could be delineated by the combination of chromosome and FISH-analyses as a constitutive heterochromatic derivate of chromosome 15. The marker was dicentric with stable inactivation of one centromere and originated from an exchange between two homologue chromosomes 15. Sperm analyses by interphase FISH showed a normal disomy rate for chromosome 15 and a proportion of the additional marker of 17.2%. ICSI-therapy was unsuccessful in the patient but lead to the birth of a healthy boy in his brother.

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DNA typeability in liquid urine and urine stains using AmpFlSTR SGM Plus

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Abstract

Purpose: Urine specimens are usually collected for biochemical and toxicological tests and for doping control. In forensic casework urine analyses are performed occasionally, however, the authors emphasize their importance in crime scene reconstruction. The objective of the research was to evaluate efficacy of AmpFlSTR SGM Plus typing of urine and urine stains which were subject to different temperature conditions.

Material and methods: Urine samples were collected from 10 female and 10 male volunteers. Liquid specimens were stored at room temperature (RT), 4°C and -20°C up to 28 days. Experimental stains were prepared by applying 3 ml urine on sterile cloth 30x30 cm, air-dried and stored at RT up to 360 days. The amount of DNA was estimated with use of slot-blot technique (Quantiblot Human DNA Quantitation Kit, Applera). DNA profiles were obtained using AmpFlSTR SGM Plus and 310 ABI Prism Genetic Analyzer (Applera). Typing of a experimental sample was considered successful when the full profile was obtained matching that of a reference sample.

Results: Significant differences in DNA yield were noted between female and male urine samples. No differences between the extraction methods were found in regard to DNA yield and typeability rate. Different typeability rates were recorded for liquid urine and urine stains depending on storage temperature.

Conclusions: Liquid urine samples and urine stains can be considered as a potential source of DNA in disputable specimen individualization and in forensic casework using the fluorescent multiplex PCR system AmpFlSTR SGM Plus.

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Introduction

Normal human urine specimens normally contain low numbers (up to 400 cells/ml) of epithelial cells (i.e. renal tubular, transitional urothelial, and squamous) [1]. Commonly, urine samples are collected for biochemical and toxicological tests and for doping control. In these circumstances, assessment of sample origin is unnecessary, unless sample switching or handling are suspected. In forensic casework urine analyses are performed occasionally, particularly in sexual assaults, therefore identification and individualisation of urine stains and samples does not pose a medico-legal concern unlike bloodstains, saliva or sperm [2], however, the authors emphasize their importance in crime scene reconstruction. The objective of the research was to evaluate efficacy of AmpFlSTR SGM Plus typing of urine and urine stains subject to different temperature conditions.

Material and methods

Urine samples were collected from healthy volunteers (10 females and 10 males). Liquid specimens were stored at room temperature (RT), 4°C and -20°C up to 28 days. In order to collect specimens for DNA extraction the latter samples were freeze/thawed every seven-days of incubation. Reference specimens were frozen once after the collection and thawed on the day 28. Experimental stains (n=20) were prepared by applying 3 ml urine on sterile cloth 30x30 cm, air-dried and stored at RT up to 360 days. Bloodstains collected from the same subjects served as reference. Urine samples of 1 ml volume were centrifuged at 13,600 x G for 5min. The supernatant was aspirated leaving 50 μl sediment. DNA samples were extracted from the liquid specimens and 2.0x2.0 cm stain cuts using Chelex 100 [3] and organic procedure [4]. The amount of DNA was

Table 1. **The typeable rates (%) of liquid urine samples using AmpFlSTR SGM Plus depending on storage temperature**

Storage period	**Male** (n=10)		**Female** (n=10)	
	Extraction			
	organic	chelex	organic	chelex
Fresh	100	100	100	100
RT (1 day)	100	100	100	100
RT (7 days)	70	70	80	80
RT (14 days)	40	30	50	50
RT (21 days)	20	10	30	20
RT (28 days)	10	10	10	10
4°C (1 day)	100	100	100	100
4°C (7 days)	100	100	100	100
4°C (14 days)	60	50	70	70
4°C (21 days)	40	30	50	40
4°C (28 days)	30	20	30	20
-20°C (1 day)	100	100	100	100
-20°C (7 days)	100	100	100	100
-20°C (14 days)	100	100	100	100
-20°C (21 days)	90	70	90	90
-20°C (28 days)	80	70	90	80

Table 2. **The typeable rates (%) of urine stains using AmpFlSTR SGM Plus depending on storage temperature**

Storage period	**Male** (n=10)		**Female** (n=10)	
	Extraction			
	organic	chelex	organic	chelex
RT (1 day)	90	90	90	90
RT (30 days)	90	90	90	90
RT (60 days)	90	90	90	90
RT (90 days)	80	80	90	90
RT (180 days)	50	50	70	60
RT (360 days)	30	20	40	30

estimated with use of slot-blot technique (Quantiblot Human DNA Quantitation Kit, Applera). 1 ng target DNA was amplified using GeneAmp PCR System 9700 (Applera) according to the manufacturer's instructions (AmpFlSTR SGM Plus PCR Amplification Kit: User's Manual, Applera). Genotyping was performed in 310 ABI Prism Genetic Analyzer using GeneScan Analysis v3.1.2 and Genotyper v2.5 software. The typing of an experimental sample was considered successful when the full profile was obtained matching that of a reference sample.

Statistical data analysis

All obtained results were statistically analysed and expressed considering average measurement error (SEM). Statistical significance of all differences in respective results was assessed using ANOVA. The level of significance was 0.05. All data were standardised for each series.

Results and discussion

Since urine contains minute amounts of nucleated epithelial cells and leucocytes, concentration of urine samples and cell sedimentation prior to DNA extraction is essential to efficient genotyping. The yield of DNA extracted from female and male liquid urine samples was 50-230 ng and 10-65 ng, respectively. The yield of DNA extracted from female and male urine stains was 1.5-10 ng and 0.3-3.5 ng, respectively. The differences were statistically significant ($p<0.05$). No differences between the extraction methods were found in regard to DNA yield and profile typeability rate. Similar results were obtained by Vu et al. [5], while Dimo-Simonin et al. [6] reported substantial differences in DNA yield between chelex and organic extraction. According to Prinz et al. [7] storage of 20 ml urine for 6 months at 4°C resulted in decrease of isolated DNA from 20-40 ng to 1-2 ng and from 400-800 ng to 10-20 ng for males and females, respectively.

The obtained typeability rates are summarized in *Tab. 1* and *2*. All fresh liquid samples were easily typeable with AmpFlSTR SGM Plus kit. For urine specimens stored at room temperature for 14 days the rate of typeable profiles decreased to 50% and below, due to absence of larger amplicons, most likely caused by DNA degradation. According to Schmitt et al. inconclusive results may result from allele drop-out due to small numbers of cells in urine specimens [8]. Other authors [7, 9, 10] found that removal of contaminants, including bacteria, from urine samples improves efficacy of DNA amplification. Liquid specimens stored at -20°C up to 28 days produced 70-90% typeability depending on extraction method and donor's sex. According to van der Hel et al. 33% of urine specimens submitted to long-term storage at -20°C yielded high-molecular weight DNA [11]. Also Dino-Simonin and Brandt-Casadevall [6] claimed that freezing is the best method of urine storage. In our material, following three freeze/thaw cycles negative effect on DNA quality was noted. Other authors indicated that repeated freeze/thaw cycles result in lysis of the urether epithelial cells in urine specimens facilitating release of nuclear DNA and its hydrolysis by endogenous nucleolytic enzymes which consequently diminishes the possible source of DNA [12,13]. For experimental urine stains stored up to 60 days the typeability rate was 90%. After 360 days typeability rate decreased to 20-40% depending on extraction method and donor's sex.

We conclude that liquid urine samples and urine stains can be considered as a potential source of DNA in disputable specimen individualization and in forensic casework using the fluorescent multiplex PCR system AmpFlSTR SGM Plus.

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Overview how adenocarcinoma cancer cells avoid immune- and chemotherapy-induced apoptosis

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Abstract

This review describes some aspects of uncontrolled tumor growth and development. In the past, it has been shown that colon adenocarcinomas use several tactics to avoid cell deletion and to maintain cell viability. In particular, colorectal cancer cells resist death ligands-induced apoptosis by expressing anti-apoptotic proteins. By direct interaction with FADD, the FLIP protein inhibits the signal transmission from death receptors to their cytoplasmic targets in COLO 205 cells. Colorectal cancer cells also stimulate own survival by inhibiting cytotoxic signals induced by interferons. Moreover, IFN-α increases immune-resistance of colon cancer cells by activation of NF-κB. Additionally, the cytoplasmic retention of proapoptotic protein clusterin also supports viability of cancer cells. Upon suitable stimulation normal cells are featured by clusterin translocation to the nucleus with concomitant cell death. We found that proapoptotic activity of clusterin is dependent on calcium ions, and depletion of intracellular calcium caused extensive death of COLO 205 cells. Other type of strategy to inhibit chemotherapy-dependent cell death is the activity of multidrug resistance proteins (MDR). These cell membrane efflux pumps actively expel the drugs from the cell interior to prevent their action on intracellular targets. The reversal of P-gp efflux pump in chemoresistant COLO 320 cell line was observed upon phenothiazine derivatives. The variety of antiapoptotic mechanisms in colorectal cancer cells makes anticancer therapy a great challenge but detailed knowledge of their complexicity gives promise to sensitize cancer cells to death stimuli.

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Introduction

Programmed cell death (apoptosis) assures every day elimination of the transformed cells to maintain regular functions of tissue organs. Apoptosis could be induced extrinsically by death ligands (TNFα, TRAIL, FasL) and/or other cytokines (IFNα, IFNβ, IFNγ) released by the immune system or intrinsically when cell death machinery is solely activated by mitochondria [1,2]. Additionally, sudden uncompensated intracellular changes in calcium homeostasis could also induce cell death [3,4]. During evolution, however, cancer cells developed and conserved several molecular mechanisms allowing them to avoid apoptotic signals. They either, gradually express less or modify membrane receptors (transmembrane ligands, soluble receptors) or they express constitutively active oncogenes. Furthermore, a plethora of antiapoptotic proteins, which inhibit death signals at different levels of signaling pathway, have been identified. Moreover, cancer cells resist chemotherapy producing a variety of proteins called multidrug resistance proteins (MDR), which act as an efflux pumps. Consequently, anticancer drugs are expelled extracellularly leading to low efficacy of the chemotherapy of cancer.

Colon cancer is one of the most common types of cancer and ranks third place in terms of occurrence and death rates among population in developed countries. The increasing number of people affected by colon adenocarcinomas in the most advanced metastatic forms prompted us to seek how this type of tumor develops to the stage where neither immune system nor chemotherapy could help. As an experimental model the human colon adenocarcinoma COLO 205 and COLO 320 cancer cell lines were used.

The resistance of colon adenocarcinoma cells to TNF-α-induced apoptosis

The TNF-α and its receptors are the best known group representing the TNF-α-superfamily death signals [5,6]. When TNF-α is released from macrophages it might interact with each of the two specific transmembrane receptors TNF-R1 and TNF-R2. However, it is TNF-R1 that plays the role to transduce the order from the immune system. In physiological conditions, if TNF-α trimeric ligand binds to its oligomerized receptor it enables to trigger few, sometimes opposite cellular responses. The response to die or to survive depends on the type of the adaptor proteins which subsequently assembly with TNF-R1 by homotypic interactions between death (DD) or death effector domains (DED) of respective proteins. Initially, TRADD (TNF-R1-associated death domain protein) is linked to intracellular domain of TNF-R1 (DD-DD) followed by assembly of FADD (Fas-associated death domain), RAIDD (RIP-associated ICE-homologous protein with death domain) or TRAF (TNF-R-associated factor) proteins to TRADD by DED-DED interactions.

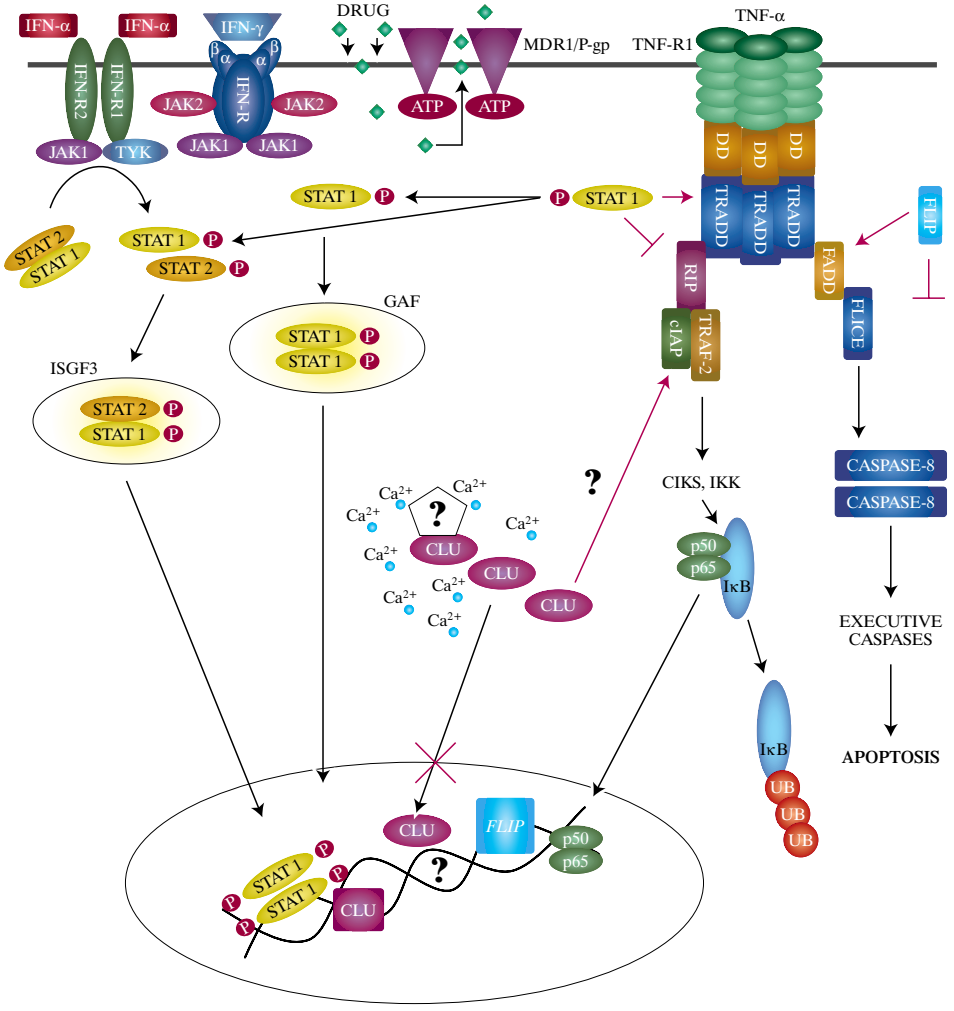
Preferentially, TRAF2 (TNF-R-associated factor-2) and RIP (receptor interacting protein) interact with TRADD (complex I) [7] to form the so-called complex I. Consequently, other important functional proteins, CIKS (NF-κB inducing kinase, NIK), are sequentially recruited to activate JNK (c-jun N-terminal kinase) or survival-signalosome by IκBα (inhibitory subunit of NF-κB) phosphorylation and subsequent NF-κB (nuclear factor kappa B) activation [8]. The latter is the transcriptional factor crucial for cell survival since it transactivates several antiapoptotic genes *A20* [9], *cIAP1/2* [10], *TRAF1/2* [11], *Bfl-1/A1* [12], *IEX-1L* [13] and *Bcl-x_L* [14]. On the other hand, if the synthesis of antiapoptotic proteins is not sufficient for the protection from apoptosis, the complex I dissociates and complex II called DISC (death-initiating signaling complex) is recruited [7]. In complex II, TRADD associates with FADD to allow FLICE (FADD-like interleukin converting enzyme) dimerization. FLICE is autocatalytically activated to caspase-8 [15] and at that moment other downstream caspases could be activated (caspase-3, -6, -7) and programmed cell death (PCD) is executed. Additionally, if RAIDD and RIP are recruited to TRADD, then procaspase-2 is activated [16]. However, the final cellular effect of TNF-α depends on the quantitative input of the particular transmission pathway. Recently, it was found, that additional interactions at the level of adaptor proteins with STAT 1 (signal transducer and activator of transcription 1) kinase or clusterin render TNF-α signal more complex and hard to predict [17,18].

In COLO 205 cells the TNF-α-induced death signal is limited to the receptor type. Irrespective to the expression of either type of receptor, solely TNF-R1 is able to interact with soluble TNF-α ligand (sTNF-α). The TNF-RII is activated by the direct interaction with anchored transmembrane TNF-α of the activated macrophages [19] but do not respond to sTNF-α. However, in our study we found, that the TNF-α does not induce apoptosis in COLO 205 cells, even though these cells express the functional TNF-RI receptor [20]. We hypothesized that some antiapoptotic protein(s) had to inhibit TNF-α-dependent death signal. The potent candidate is cFLIP (FLICE-inhibitory protein) protein, which competes with FLICE for a binding in the DISC complex. There are known two isoforms of cFLIP: $cFLIP_S$ and $cFLIP_L$. On one hand, $cFLIP_L$ is structurally similar to procaspase-8, since it contains two DEDs and a caspase-like domain (CASP). However, the latter domain lacks residues that are important for the catalytic activity of caspase-8 [21,22]. On the other hand, $cFLIP_S$ possesses only two DEDs. Via DED--DED interaction cFLIP is recruited to FADD protein and activation of caspase-8 is blocked. As a regulator of lymphocytes T and B maturation and embryonic cells development also positive effects of cFLIP were reported (for details see the review article by Pajak et al. [23]).

Importantly, the presence of cFLIP has been identified in many types of cancer cells and is proposed to cause the resistance to death ligand-induced apoptosis [24-27]. To verify the role of cFLIP in cancer cell apoptosis the mouse lymphoma MBL2-Fas cancer cells, which express the high level of Fas protein and tumor embryonic AR6 cell, with low level of Fas, were both transfected with cFLIP protein [28]. Transfected and mock-transfected cells were injected into mice, however, exclusively cFLIP-transfected cells (MBL2-Fas and AR6) developed into cancer. Interestingly, the presence of cFLIP (antiapoptotic) but not Fas protein (proapoptotic) appeared crucial for cancer development. Importantly, the overexpression of $cFLIP_L$ was found in colon carcinoma cells isolated from surgical specimens [29]. The promising approach is limiting cFLIP protein level, once achieved it could sensitize cancer cells to death ligand-induced cell death. Actually, it has been shown that the use of metabolic inhibitors, such as anisomycin, emetine, harringtonine or puromycin [26] sensitize prostate cancer cells to TRAIL-induced apoptosis. Similarly, the treatment with actinomycin D (transcription inhibitor) and/or cycloheximide (CHX) (non-specific translation inhibitor) of immune resistant cancer cells render them sensitive to FasL [30,31].

In our studies cycloheximide, when used alone, did not cause cell death at least during initial 12 hours, however, when cells were treated with TNF-α in the presence of CHX, the viability of COLO 205 adenocarcinoma cells progressively and inevitably fall down. It was confirmed by electron microscopy analysis that COLO 205 cells died from the extensive apoptosis [20]. The Western blot and immunocytochemistry analyses showed that the cFLIP protein level decreased although it was not the case when TNF-α was used alone. Moreover, the immunoprecipitation studies confirmed that the cFLIP level dropped in DISC complex, and that this reduction was accompanied by the higher caspase-3 activity. The results seem to support the hypothesis, that COLO 205 cancer cells could no longer avoid TNF-α--induced apoptosis if protein synthesis is inhibited. To clarify the exact role of cFLIP in TNF-α signaling pathway we used siRNA oligonucleotides to knockdown FLIP gene. It was supposed that silencing FLIP gene would resemble CHX co-treatment. In fact, western blot analyses demonstrated decreasing levels of cFLIP in siRNA transfected cells (in comparison to mock-transfected cells). Additionally, TNF-α treatment activated procaspase-3 in siRNA transfected COLO 205 cells (unpublished data). These results confirm the statement that cFLIP retards

Figure 1. **Schematic representation of the molecular mechanisms involved in "immune escape" and chemoresistance of colon adenocarcinoma cells. STAT 1 kinase plays significant role by inhibiting both the formation of TNF-R1 complex I and subsequent NF-κB activation. IFNAR and IFNGR compete with TNF-R1 for STAT 1. Thus, IFNs release TNF-R1 complex I from STAT 1 kinase inhibition. Calcium ions (Ca^{2+}) sequester clusterin (CLU) in the cytoplasm and by this route COLO 205 cells become resistant to apoptogenic stimuli. Lastly, high expression of P-gp efflux pump renders COLO 320 cells unsusceptible to anticancer drugs. Black arrows indicate the direction of the respective signaling pathways. Red arrows indicate targets for possible interactions whereas dashed stops show subsequent blockages**



TNF-α-induced death signal. Because TNF-R1 DISC complex is similar to DISC complexes found at DR 4 and DR 5 receptors activated by TRAIL (TNF-α-related apoptosis inducing ligand), it is assumed that cFLIP expression might also help COLO 205 cells to avoid TRAIL-induced apoptosis [32]. However, hitherto no available data exist that confirm the role of cFLIP in TRAIL-induced apoptosis in COLO 205 cell line.

The crosstalk between interferons and TNF-R1 signaling pathways in COLO 205 cells

Interferons (IFNs) are a family of cytokines of multifunctional activity. They block viral infections, inhibit cell proliferation and modulate cell differentiation [33]. These cytokines also play important roles in immunosurveillance for malignant cells. The IFNs family includes two main classes of related cytokines (type I and type II). There is a variety of type I interferons, all of which have considerably similar structure. In humans these group include IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω [34,35]. All type I IFNs assembly with a common cell surface receptor, which is known as the type I IFN receptor (IFNAR) [34]. By contrast, there is only one type II interferon called IFN-γ that binds another cell surface receptor known as the type II IFN receptor (IFNGR) [35]. The type I receptor is composed of two subunits, IFNAR1 and IFNAR2, which are associated with the Janus activated kinases (JAKs): tyrosine kinase 2 (TYK2) and JAK1, respectively. The type II receptor is also composed of two subunits: IFNGR1 and IFNGR2, which are in turn associated with JAK1 and JAK2, respectively. Activation of JAKs by type I IFNs results in tyrosine phosphorylation of STAT 2 and STAT 1; this leads to the formation of STAT 2-STAT 1-IRF9 (IFN-regulatory factor 9) complexes, which are also known as ISGF3

(IFN-stimulated gene factor 3) complexes. These complexes translocate to the nucleus and bind IFN-stimulated response elements (ISREs) in DNA strand to initiate gene transcription. Both types of IFNs also induce the formation of STAT 1-STAT 1 homodimers that translocate to the nucleus and bind GAS (IFNγ-activated site) elements that are present in the promoter regions of certain ISGs, thereby initiating the transcription of these genes [36-39].

It is well established that IFNs induce the expression of hundreds of genes, which mediate various biological responses. In any case, it has been apparent that the activation of JAK--STAT pathways alone is not sufficient for the generation of all of the biological activities of IFNs. There is accumulating evidence that several other IFN-regulated signaling elements and cascades are required for the generation of many cellular responses to IFNs. Some of these pathways co-operate with JAK--STAT pathway, whereas others only with STATs. For example, type I IFNs were shown to activate the mitogen-activated protein kinase (MAPKK) p38 and its downstream effectors [40]. Moreover, also the activity of PI3-K signaling pathway could be modulated by type I IFNs [41,42]. In cancer cells IFNs have been examined as potential synergistic agents in death ligands-induced apoptosis [43,44]. STAT 1 kinase was also described as a component of TNF-R1 signaling complex. According to Wang et al. [45] in HeLa cell line stimulated with TNF-α the STAT 1 kinase binds to TRADD protein and attenuates the interaction of TRADD with TRAF2 and RIP. As a consequence the NF-κB activation is blocked. The authors claimed that the TRADD-STAT 1 complex promotes DISC formation and TNF--α-dependent apoptosis. In our studies performed on COLO 205 cells we found that neither IFN-α nor IFN-γ, and not even IFN-α combined with IFN-γ treatment affect cell survival (unpublished data). Interestingly, also NF-κB activation in the presence of TNF-α was not detectable [18].

We hypothesized, that the lack of NF-κB activation results from the presence of STAT 1 kinase in TNF-R1 complex. The immunoprecipitation analysis confirmed this interaction. Moreover, the level of STAT 1 kinase in TNF-R1 signalosome was TNF-α-dose-dependent [18]. We also concluded that inefficiency of IFNs to amplify death signal might be a consequence of the competition for STAT 1 kinase between IFNs and TNF-α signaling pathway. Again, the immunoprecipitation studies showed that in the absence of activation of TNF-R1, STAT 1 kinase is bound to TRADD protein. It seems likely, that in COLO 205 cells, STAT 1 kinase preferentially binds TNF-R1 complex. Consequently, the decreased level of STAT 1 in the cytoplasm limits the transduction of IFNs signal. The pretreatment of COLO 205 cells with IFN-α or IFN-γ reduced the STAT 1 kinase level in TNF-R1 complex. IFN-α also stimulated Y701 phosphorylation and increased the expression of STAT 1. Regardless of the reduced level of STAT 1 complexed with TRADD, only IFN-α pretreatment combined with subsequent administration of TNF-α subsequently induced NF-κB (unpublished data). Lack of STAT 1 phosphorylation by IFN-γ suggests the disturbances in the upstream kinases activity, probably JAK2. It is also not clear whether NF-κB activation was induced by TNF-α or whether it resulted from the higher PI3-K activity stimulated by type I IFNs (at the moment this aspect is under scrutiny). It is clear from this examination that administration of IFN-α could stimulate colon cancer cell survival. In the past, the conflicting results led to the concern whether there is sufficient evidence to make use of IFN-α in cancer therapy. There are known positive effects of type I IFNs action against hematopoietic neoplasms [46,47] and some vascular tumors, such as pulmonary hemangiomatosis, infantile hemangiomas, Kaposi's sarcoma and others [48]. IFN-α was also effective in some groups of patients suffering from renal cancer [49] and melanoma. At the same time, there are reports indicated negative effects of type I IFNs in anticancer treatment [50,51]. Our observations are in favour of the opinion stressing possible negative effect of IFN-α therapy in colon cancer therapy.

The calcium-dependent regulation of apoptotic activity of clusterin

As indicated in the first section of the article, apoptosis is induced by extrinsic signals but could be also activated by intrinsic pathway. One of the most important modifiers of apoptosis is calcium homeostasis. Changes in calcium ion concentration within different cell compartments activate pro- or antiapoptotic proteins. It is known, that there are some types of cancers which have increased intracellular calcium level in comparison to normal cells. According to our results, the reduction of intra- but not extracellular calcium concentration reduces COLO 205 cell survival [20]. Among the antiapoptotic proteins blamed for this phenomenon, clusterin (CLU) is a potent candidate. The exact function of clusterin in the regulation of cell death remains unclear, especially in cancer cells, although, according to the latest reports, clusterin could play significant role in the failures of chemotherapy and/or modulation of immune system to delete cancer cells. Clusterin has a wide range of physiological functions (for details see the review by Pajak et al. [52]), however, clusterin has been described also as both a pro- and as an antiapoptotic protein. The discrepancy in function of CLU could be related to the specific proteomic profile, which results from the cell type specific routes of expression CLU. The main product of clusterin gene expression is 60 kDa precursor form which is glycosylated and cleaved to α- and β-subunits, held together by five disulfide bonds [53]. The mature, 80 kDa protein (sCLU) could be secreted to the extracellular space and body fluids [54]. The second 50 kDa isoform of clusterin (intracellular iCLU) is localized in the cytoplasm. Upon some cytotoxic stimuli, including ionizing radiation (IR) [55], transforming growth factor β [56], phorbol ester (TPA) [57], or cytokines, such as TNF-α [58] iCLU is activated and translocates to the nucleus. It is also indicated that clusterin activity is dependent on the type of chemotherapeutic agent. According to Scaltriti et al. [59] and Caccamo et al. [60] the administration of topoisomerase II inhibitor – etoposide, elevated the level of clusterin within the nucleus with the concomitant reduction of iCLU pool in the cytoplasm of the PC-3 prostate cancer cells. Interestingly, in adenocarcinoma COLO 205 cancer cells, etoposide induced the nuclear localization of iCLU, but the Western blot analysis did not show the decreased level of 50 kDa clusterin in the cytoplasm (unpublished data). The presence of clusterin in the COLO 205 cells

nuclei could result from changes in gene transcription profile as an alternative to CLU translocation.

In 2005, Caccamo et al. [61] presented interesting observations pointing to the role of calcium ions in iCLU activity. The use of BAPTA-AM (intracellular calcium ions chelator) in PC3 prostate cancer cells resulted in a highly significantly inhibition of both cell growth and viability. Cell death was associated with characteristic hallmarks of anoikis. The accumulation of iCLU in the nuclei was accompanied by the activation of execution caspases and progression to apoptosis. Our results obtained from COLO 205 cells are similar, although we independently used other calcium chelators. Diminishing extracellular calcium concentration by EGTA in COLO 205 cells did not induce changes in iCLU localization, whereas EDTA, which chelates both extra- and intracellular calcium, promoted nuclear iCLU expression with concomitant caspase-3 activation. These results suggest that the lack of iCLU in the nuclei of colon adenocarcinoma cells promotes cell survival, whereas its translocation/expression within nucleus correlates with cell death (article in press). It is not clear, however, what are the details of proapoptotic activity of CLU. Keeping in mind the presence of coiled-coil domain in the clusterin sequence (domain characteristic for some transcription factors) we suppose that CLU is able to interact with DNA to control the expression of proapoptotic genes. The *in vitro* observations have been confirmed by the *ex vivo* studies, which also demonstrated the increased level of nuclear localization of iCLU in tumor-bearing gut compared with normal subjects [62]. The analogous results were obtained from prostate, breast and colon cancer specimens suggesting the existence of a common molecular mechanism of CLU action in cancer cells. The knowledge about clusterin activity and its possible regulators, such as calcium channel modifiers, could be a promising prospect in future cancer therapy. The latter studies are also needed to explain the connections between clusterin and the TNF-α signaling pathway, especially if the effect of calcium chelators does not oppose cell viability affected by cycloheximide and TNF-α [18].

MDR proteins and the resistance to chemotherapy-induced cell death

Since cancer cells avoid immune system-induced apoptosis, the presently used cancer treatments are often based on administration of compounds which promote physical or chemical stress in order to evoke apoptosis through the mitochondrial or intrinsic pathway. However, chemotherapy is effective only in some cancer types. In addition, even if tumors initially are not resistant to a specific anticancer treatment, the genetic and epigenetic heterogeneity in the face of the powerful selection imposed by potent anticancer drugs results in overgrowth of drug-resistant variants and the rapid acquisition of drug resistance by many cancers. A great deal of colon cancers confers simultaneous resistance to many different structurally and functionally unrelated drugs. This phenomenon, known as multidrug resistance [63], can results from changes that limit accumulation of drugs within cells by limiting uptake, enhancing efflux, or affecting membrane lipids [64]. Such changes unable the execution of programmed cell death previously activated by anticancer drugs administration. It came as something of a surprise that the major mechanism of multidrug resistance in cultured cancer cells was the expression of an energy-dependent drug efflux pump, known alternatively as P-glycoprotein (P-gp) or the multidrug transporter [65]. The P-gp, the human *MDR1* gene product is one of the 48 known ABC transporters in human cells. It is also highly expressed in cancers of gastrointestinal origin (small and large intestine, liver cancer and pancreatic cancer), cancers of hematopoietic system (myeloma, lymphoma, leukemia), cancers of genitourinary system (kidney, ovary, testicle), and childhood cancers (neuroblastoma, fibrosarcoma). P-gp is a protein that can detect and bind a large variety of hydrophobic natural-product drugs as they enter the plasma membrane. These drugs include many commonly used anticancer drugs such as doxorubicin and daunorubicin, vinblastine and vincristine, and taxol, as well as many pharmaceuticals. Binding of these drugs results in activation of one of the ATP-binding domains, and the hydrolysis of ATP causes a major change in the shape of P-gp, which results in release of the drug into the extracellular space [66].

The current efforts are focused on identification of new compounds, which by direct interaction with P-pg could inhibit its drug-transport activity *in vitro* in experimental models [67]. Hitherto, wide ranges of natural and newly synthesized compounds have been described as potent inhibitors of P-gp activity. As an effective in inhibition of drug efflux carotenoids, flavonoids and other natural plant derivatives were described [68]. Phenothiazines are also among the compounds known to modify MDR mediated by P-gp in various cancers. In our studies on MDR/COLO 320 cell line the perphenazine and prochlorperazine, the new phenothiazine derivatives, appeared as potential inhibitors of drug efflux [69]. The combined treatment of cytotoxic drugs with concomitant administration of MDR inhibitors seems to be a potential approach in the future cancer therapy.

Conclusions

Antiapoptotic strategies developed by colon cancer cells prevent their proliferation and lead to cancer progression. The first level of cancer resistance is the "immune escape" – the inhibition of death signals induced by natural killers, such as death ligands and interferons. The insufficiency of TNF-α to induce extrinsic apoptosis is caused by the overexpression of the antiapoptotic proteins, such as FLIP, which inhibit the death signal transduction. Therefore, the administration of metabolic inhibitors, which limit the level of antiapoptotic proteins, could sensitize cancer cells to natural apoptogenic signals. Unfortunately, the clinical use of metabolic inhibitors has been limited because of their non-specificic action and toxic side effects. The new natural, non-toxic compounds are intensively tested including modulators of P-gp transport proteins. On the other hand, the interferons, which were sometimes reported as promising in anticancer therapy, appeared totally inefficient to potentiate TNF-α-dependent death of COLO 205 cells. Moreover, the IFN-α treatment might activate antiapoptosis by NF-κB-dependent genes and by the involvement of STAT 1 kinase

in both signaling pathways. So far, the crosstalk between TNF-α and IFNs transduction pathways has not been described in details, but it seems to be one of the most significant achievements of molecular evolution in colon cancers. Clarification the role of clusterin role in TNF-α-dependent signaling pathways as well as possible function of STAT 1 kinase in transcription of antiapoptotic genes in cancer cells could widen the perspectives for search of a new anticancer strategies. New findings related to the molecular mechanisms of antiapoptosis could show new targets for future anticancer therapy. Moreover, the sequential treatment with metabolic inhibitors, death ligands (TRAIL) and modulators of calcium homeostasis (such as calcium channels modifiers or calcium chelators) might be a promising tool to delete cancer cells whereas the inhibitors of drug efflux might be beneficial to minimize the losses of active substances in anticancer therapy.

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Levels of HBV-DNA, sFas and sFasL among healthy HBsAg carriers in period of three years

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Abstract

Purpose: The object of the study was the usefulness of sFas and sFasL concentration in the prognosis of disease development in healthy HBsAg carriers.

Patients: 34 healthy HBsAg carriers were examined over a three-years period.

Material and methods: HBV-DNA was extracted using the Gene Elute Mammalian Genomic DNA Miniprep Kit (Sigma, USA). HBV-DNA concentration and YMDD mutations were measured by RT-PCR based on TaqMan Universal Master Mix (Applied Biosystems, USA). HBeAg and anti-HBe in serum were detected by MEIA method (ABBOTT, Germany). The concentration of sFas and sFasL in serum was estimated by ELISA method (Bender MedSystems, Austria).

Results: Within three year observation period the number of carriers with absent HBV-DNA increased from 19% to 33%. HBV-DNA above 105 copies/ml, which was detected in 63% of carriers, decreased to 11% ($p<0.05$). After 3 years, a reduction of HBV-DNA levels was observed in 89% of carriers ($p<0.05$).

The occurrence of sFasL decreased from 56% to 48%. sFasL correlated with HBV-DNA ($p<0.05$). The concentration of sFas decreased ($p<0.01$). Chronic hepatitis B developed in 11% of men carriers, and 11% eliminated HBeAg, anti-HBe and HBV-DNA. YMDD mutant was not detected in any of the HBsAg carriers.

Conclusions: High concentration of sFasL in serum may suggest the development of chronic hepatitis and it seems that sFasL detection is never a good prognostic factor.

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Introduction

According to National Institute of Health [1], HBV-DNA above 105/mL in serum of chronically HBV infected persons is the basic condition of the diagnosis of chronic hepatitis B. Such diagnosis does not demand increased ALT activity, or other abnormal biochemical indicators of liver damage, to be discovered. Among persons infected with YMDD mutant HBV, lower HBV viral load in comparison to the one discovered in persons infected with the "wild" virus is responsible for the development of chronic hepatites [2]. Limited studies determining the correlation of HBV viraemia with histological changes in liver have shown a possible relation between those two factors. However, besides the level of viral load, immunity is also of significant importance in the development of changes produced by HBV infection.

The death of hepatocytes in HBV infection is the consequence of inflammatory necrotic changes or processes of programmed cell death. In HBV infection, programmed cell death is initiated mainly by the activation of Fas "death domain" present on hepatocytes. It seems that the processes of programmed cell death play a significant role in the development of chronic hepatitis [3,4].

In our previous studies [5] performed in healthy HBsAg carriers, HBV replication of above 105/mL was detected in 65% while the concentration of sFas was lower in relation to its concentration in patients with chronic hepatitis B. Current studies performed 3 years after the original analysis are the continuation of the undertaken issue and their main aim is to determine the importance of HBV viral load and the concentration of programmed cell death indicators in HBsAg carriers' state. Moreover, the rate of YMDD mutant presence in HBsAg carriers was also determined in the current studies.

Material and methods

The study included 34 HBsAg healthy carriers, 15 women (aged 20-43 yrs) and 19 men (aged 21-38 yrs) in 2002 year. The examinations were re-performed in 27 persons, 12 women (aged 23-43 yrs) and 15 men (aged 24-39 yrs) after 3 years.

Inclusion and exclusion criteria of healthy HbsAg carriers were: age 18 and over, HbsAg presence in serum for at least 1 year, normal range of ALT, AST, bilirubin, albumin and prothrombin within 1 year, unremarkable changes in USG liver examination, lack of past or present history of drug or alcohol abuse, autoimmune disorders, HCV infection, immunomodulation treatment.

Informed consent was obtained from each patient and the Bioethics Committee at the Medical Academy of Białystok approved the study protocol.

Methods

HBsAg, HBeAg and anti HBe in serum were detected by MEIA test (ABBOTT, USA).

Extraction of HBV-DNA from patients' sera

HBV-DNA was extracted from 200 ml of patients' serum using kits for DNA isolation: the Gene Elute Mammalian Genomic DNA Miniprep Kit (Sigma, USA). The part of HVB-DNA were amplificated by PCR system with primer complementary to conservative part of genome (sense 5'-AG GGG AGG AGA TTA GGT TAA-3' antisense 5'-AGG AGT GCG AAT CCA CAC TC-3') in 20 ml reaction mixture: 200 mM dNTPs, 0.4 mM all primers, 1.5 mM $MgCL_2$, 1.0 U Taq polymerase (Sigma) and 4 ml DNA solution. 40 cycles amplification was performed (in 96°C for 30 s, in 57°C for 60 s and in 72°C for 60 s). The products of amplification were appointed in 2% agar gel by electrophoresis, and next stained with ethidine bromide. Electrophoregrams were visualized in system of record and computer analyses UVI-KS400i/ /Image PC (Syngen Biotech, USA). Part DNA solution was kept in temperature -20°C for further stages of work.

HBV-DNA concentration in sera was evaluated by real-time detection PCR based on TaqMan chemistry. Amplification was performed in 25-ml reaction mixture containing 2 x TaqMan Universal Master Mix (Applied Biosystems, USA) with uracil N'-glycosylase, 30 pmol of forward primer, 30 pmol of reverse primer, 30 pmol TaqMan probe (5'-FAM) and 5 ml of isolated DNA. After incubation for 2 minutes at 50°C, which enables uracil N'-glycosylase to inactivate possible contaminating amplicons, incubation for 10 min at 95°C allowed AmpliTaq Gold polymerase to activate and inactivate the uracil N'-glycosylase. Next cycles PCR were moved. Number of copies was counted by interpolation from definite standard curve. The detection limit of this system was as few as 10 HBV-DNA copies/ml of serum. A linear standard curve was obtained between 10 and 108 DNA template copies/reaction.

YMDD mutation

Mutation of gene of virus's polymerase was performed with use of reagents "Blood Mini" (A&A Biotechnology, Poland). DNA solutions about positive results of amplification were investigated mutatory analysis with use of direct sequenced technique. Before of sequention reaction, the part of gene of virus's polymerase with rt180 and rt204 code became duplicated in single PCR or nested-PCR reaction in dependence from concentration HBV-DNA in studied sample. Amplification were performed in 20 μl mixture contain 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM every from dNTPs, 0.5 mM every from primers, 1 U DNA polymerase and 5 ml of solution of DNA, what responded of 5 ml patients serum. Amplification on road single PCR were realized with primer 840 (5'-ACCCCATCTTT TTGTTTTGTTAGG-3') and 377 primer (5'-GGATGTGTCTGCGGCGTTT-3'). In nested-PCR reaction external primer 12F (5'-AGACTCGTGGTGGACTTCTCT-3') and 5RC (5'CAAAAGAAAATTGGTAACAGCGGTA-3'), as well as internal primers 840 and 377 were used.

Amplification in thermocycler GeneAmp PCR System 2400 (Applied Biosystems, USA) having accord to following thermal profile: 5 min in 94°C, 40 cycles for 30 s in 94°C, 30 s in 55°C and 60 s in 72°C and 5 min in 72°C were executed. Identification of PCR products was executed in agar elektroforesis and dying brom etydyne.

Cleaning of PCR products before of seuquentione reaction were prepared with reagents Clean-Up (A&A Biotechnology Poland).

Reaction of sequence were executed cyclic method, in 10 μl of reactionary mixture from primers sensible 377, using finished kid of BigDye Apprentice Termister Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). Products of sequenced were cleaned by Ex Terminator Kit (A&A Biotechnology, Poland).

The sequencing was performed ABI PRISM 377 analisator (Applied Biosystems, USA) and use of software Sequencing Analysis 3.4.1.

sFas and sFasL

The sFas and sFasL concentration in the serum was measured twice by immunology – enzymatic assay test (ELISA, Bender MedSystems, Austria) [6].

Statistical analyses

Statistical analysis was performed by use of non-parametric Mann–Whitney U and Spearman tests. Values of $p<0.05$ were considered to be significant.

Results

HBV-DNA was not detected in 5/34 (19%) persons in the year 2002 and in 9/27 (33%) in the year 2006. The number of persons with HBV-DNA above 105 copies/ml, decreased from 17/27 (63%) in the year 2002 to 3/27 (11%) in the year 2006. A significant reduction of HBV viraemia after 3 years of observation was discovered in 89% of carriers, which is comparable to the reference data (*Fig. 1*).

In the year 2002, sFasL presence in serum was detected in 19/34 (56%), after 3 years of observation in 13/27 (48%) HBsAg carriers (the protein was not detected in the group of healthy persons). The rate of sFasL presence (studies from the years 2002 and 2006) correlated with high HBV viraemia ($r=0.349$, $p<0.05$). No relation between the detection of sFasL in serum

Figure 1. **YMDD mutant infection was not discovered in any of the HBsAg carriers**

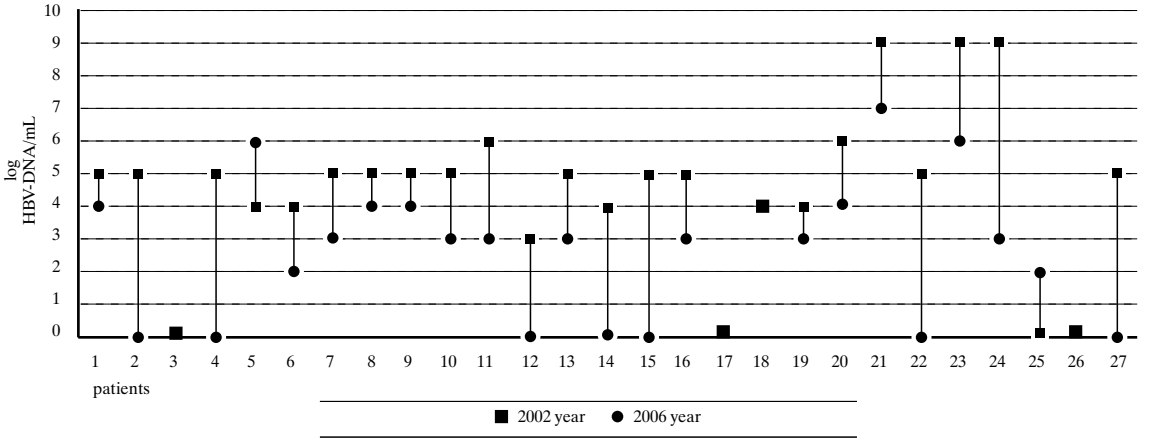
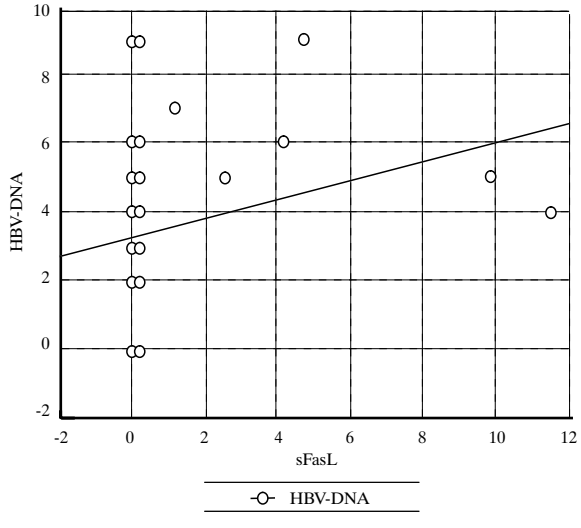


Table 1. **HBV-DNA and sFas concentration as well as percentage of sFasL in haealthy HBsAg carriers**

	n – patients (%)		**sFas concentration** (pg/ml)		**percentage of patients with sFasL** (%)	
year	2002	2006	2002	2006	2002	2006
healthy (n=12)				18.4	Absent	
healthy HBsAg carriers	34 (100)	27 (100)	15.4	12.6	19/34 (56)	13/27 (48)
HBV-DNA >105/mL	22 (65)	3 (11)	16.3	9.3	12 (35)	3 (11)
HBV-DNA <105/mL	5 (15)	15 (56)	12.9	12.9	4 (12)	8 (29)
HBV-DNA =0	7 (20)	9 (33)	14.1	13.1	3 (9)	2 (7)
HBeAg positive patients	10 (29)	2 (7)	15.8	10.0	4 (12)	2 (7)
HBV-DNA >105/mL	8 (23)	2 (7)	16.0	10.0	4 (12)	2 (7)
HBV-DNA <105/mL	0	0	0	0	0	0
HBV-DNA =0	2 (6)	0	14.8	0	0	0
anti-HBe positive patients	24 (71)	22 (81)	15.1	13.0	15 (44)	10 (37)
HBV-DNA >105/mL	14 (41)	1 (4)	16.5	8.0	8 (23)	1 (4)
HBV-DNA <105/mL	5 (15)	15 (55)	12.9	12.9	4 (12)	8 (29)
HBV-DNA =0	5 (15)	6 (22)	13.9	14.4	3 (9)	1 (4)

Figure 2. **The concentration of sFas after 3 years of observation underwent a significant reduction (Spearman test, p<0.01). No correlation between the concentration of sFas and HBV-DNA viraemia was observed despite the fact that both the HBV viral load and the concentration of sFas underwent a reduction**



and the presence of HBeAg or the anti-HBe antibody was discovered (*Tab. 1*, *Fig. 2*).

Chronic hepatitis B developed in 3/27 (11%) men carriers. After 3 years of observation, the increase in ALT activity was discovered in 3/27 (11%) men carriers. In the mentioned persons, after a morphological examination of liver tissue, clinical chronic hepatitis B was diagnosed (*Tab. 2*).

Discussion

The reduction of HBV viral load in most carriers within the period of 3 years confirms the importance of natural immunological processes in HBV infections [7]. The presence of sFasL in sera of HBsAg carriers indicates HBV stimulated expression of the domains present on lymphocytes responsible for the recognition of infected cells. In own studies, the correlation between HBV-DNA and the level of sFasL presence was detected, which indicates the importance of the processes of programmed cell death in the elimination of HBV infection. A high concentration of sFasL together with high HBV viral load seems to be a risk prognostic factor of a chronic inflammatory process. The study of Han at al. [8] proved that in comparison to patients with

Figure 3. **Within the 3-year period of observation 8/27 (30%) of carriers eliminated the HBeAg and 2/27 (7.4%) eliminated HBV-DNA**

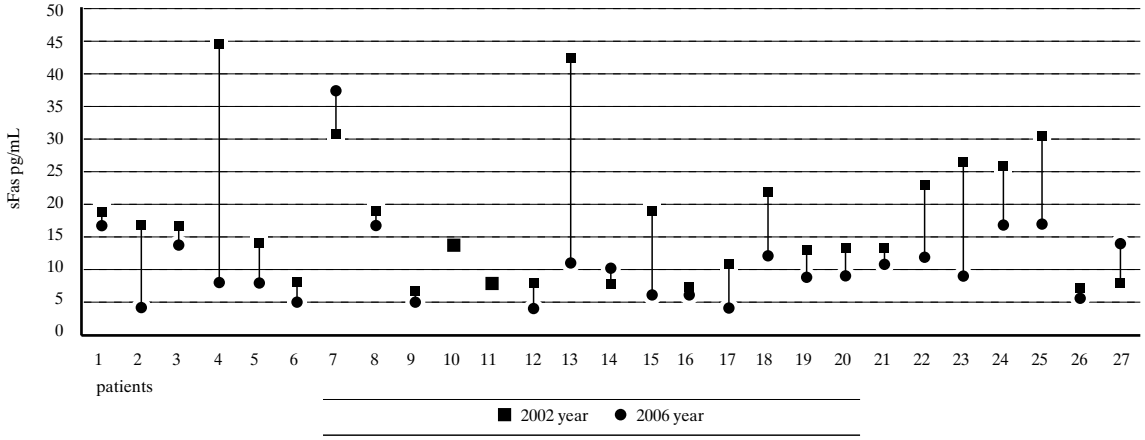


Table 2. A) three healthy HBsAg carriers which eliminated HBV-DNA, HBe and anti-HBe; B) three HBsAg carriers which followed chronic hepatitis B

A)

Person	Aged – years	HBV-DNA/mL		HBeAg		anti HBe		sFas (pg/mL)		sFasL (pg/mL)	
		2002	2006	2002	2006	2002	2006	2002	2006	2002	2006
C-M	26	1.33 x 105	0	+	-	-	-	19	6	0	0.14
K-H	31	1.01 x 1059	0	+	-	-	-	23	12	0	0
L-A	28	1.10 x 105	0	-	-	+	-	7.5	14	0	0

B)

Person	Aged – years	HBV-DNA/mL		HBeAg		anti HBe		SFas (pg/mL)		SFasL (pg/mL)	
		2002	2006	2002	2006	2002	2006	2002	2006	2002	2006
M-P	24	9.09 x 104	6.12 x 106	-	-	+	+	14.0	8.0	11.5	4.16
B-S	27	1.93 x 109	1.14 x 107	+	+		-	13,0	11.0	4.73	1.15
M-O	24	1.39 x 105	5.34 x 103	-	-	+	+	43.0	11.0	9.89	0.09

chronic hepatitis B and those with liver cirrhosis the concentration of soluble TRAIL (TNF-related apoptosis inducing ligand) molecules in HBsAg carriers is lower. The results of the studies, similarly as in the studies being presented, indicate the existence of a relation between high concentration of the indicators of programmed cell death, such as sTRAIL, and the exacerbation of liver damage. This is confirmed by the observed development of chronic hepatitis in carriers with such type of parameters. This is important because in most carriers with HBV viral load above 105 copies/ml and low concentration of sFasL (or the absence of the protein in serum), after 3 years of observation no traits of the disease development were detected and the HBV viral load underwent a reduction.

The concentration of sFas, similarly to viral load, decreased within the period of a few years' observation. Such result can indicate a reduction of programmed cell death activity in the states of hypostimulation of these processes by viruses. A reduction of viral load and the concentration of sFas is a very good indicator, indicating HBV infection being reduced. The studies of Xin at al. [9] confirm the relations between the concentration of Fas in serum and liver tissue and the activity of apoptosis whereas the studies of Hayashi and Mint [10] confirm the coexistence of the elimination of infected hepatocytes and cytotoxic activity.

It was not proved in own studies that seroconversion in HBe system influences the elimination of HBV, however, the virus replication was observed. At present, it is hard to determine whether the elimination of HBV together with the appearance of anti-HBe is a prognostically good factor. The studies of Chu at al. [11] confirm this suggestion.

Antiviral treatment of HBsAg carriers with viral load above 105 copies/ml is justifiable, which is also confirmed by own studies. YMDD mutant (514C-A, 523C-A, 562T-A or 667C-A) was not discovered in any HBsAG carrier. Peng and all. [2], examining HBV infected patients with the presence of anti-HBe antibodies and with viral load from 10 to 105 copies/ml showed the presence of precore mutant 1 869G-A in 86.9%, and mutant 1 762A-T/1 764G-A in 32.8% persons. The results of these studies indicate frequent mutations in the case of HBV infections and as it is widely known the mutants of this virus can cause significant liver damages even in the case of low viral load. It is expedient thus to use such prognostic factors that will enable to make a decision of treatment of HBV infected patients despite detecting low viral load. It seems that the examination of the presence and concentration of sFasL simultaneous with the examination of viral load can facilitate the initiation of antivirus treatment.

Conclusions

The concentration of sFas and the rate of sFasL presence (the indicators of programmed cell death) correlate with HBV-DNA among HBsAg carriers. High HBV-DNA viral load together with high concentration of sFasL in serum may suggest the development of chronic hepatitis. The absence of sFasL in serum seems to be the factor of the elimination of HBV-DNA.

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Biomarkers in clinical practice: a tool to find subjects at high risk for stomach cancer. A personal view

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Direct diagnosis of gastric cancer with a single laboratory test is impossible. Gastric biomarkers assayed from a blood sample provide, however, a tool to diagnose patients at particularly high risk for gastric malignancy. This possibility is commonly neglected although the biomarkers provide a handy and inexpensive way to rationalize the clinical practices, and to identify the subjects most likely to have a gastric neoplasia, and in whom a careful immediate diagnostic endoscopy is most beneficial.

Helicobacter pylori gastritis and atrophic gastritis precede gastric cancer in 50-80% of the cases, and atrophic gastritis is inevitably the most important single risk condition of gastric cancer known and identified so far [1,2]. Strategies intended to find the subjects with this preceding condition with blood tests followed by diagnostic endoscopy have provided promising results in early diagnosis of the gastric malignancy [3]. Subjects with atrophic gastritis have a 2-90-fold risk of gastric cancer compared to subjects with normal and healthy stomach (normal gastric mucosa: no inflammation, no atrophy, no *H. pylori*) [2-4].

The risk of gastric cancer increases with increasing grade and extent of atrophic gastritis in the stomach, and is highest in those with severe atrophy [1,2]. Assays of the levels of serum pepsinogen I (S-PGI) and II (S-PGII), and gastrin-17, as well as *H. pylori* – antibodies provide a possibility to diagnose the subject with atrophic gastritis, and to establish with high sensitivity and specificity in which part of the stomach the atrophic alterations are, and how extensive the atrophy is. On the other hand, the blood test with biomarkers also reveals with high accuracy those who have a normal and healthy stomach and in whom the cancer risk, correspondingly, is practically nil [5-8].

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In two Finnish cross-sectional population-based studies on "asymptomatic" subjects, gastric cancer or its early stage was found in 4-6% of the 50-65 year-old men who showed a moderate or severe atrophy of the corpus mucosa with the S-PGI test [3]. The atrophy was diagnosed by screening more than 20,000 men and the gastroscopy was performed to 1,344 men who had a low serum level of PGI (S-PGI <25 μg/l). Of these, 80% had *H. pylori* antibodies, and 63 out of 1,344 men had gastric cancer or cancer preceding lesion (dysplasia, intramucosal neoplasia). The study demonstrated further that approximately 70% of the cancers identified in the screening program were at early stage ("early cancers"), all of which patients could be curatively healed by the surgery or endoscopic mucosectomy. As compared to findings in endoscopy and histology, the sensitivity and specificity of the low PG I level (<25 microg/l) were 78% (95% confidence interval 75-80%) and 98% (95-100%) for advanced (moderate or severe) atrophic gastritis.

Approximately 5-10% of Finnish males at age over 50 have an advanced (moderate or severe) atrophic gastritis of the corpus which may lead, in addition to gastric cancer, to low output of intrinsic factor and consequently to malabsorption of vitamin B_{12} [9,10]. Of the patients with advanced atrophic corpus gastritis, 30% have an exceptionally low (<170 pmol/l) levels of vitamin B_{12} in serum, and 50% have the vitamin levels <220 pmol/l that typically associates with increased serum levels of homocysteine [10]. On the basis of the available prevalence rates of atrophic gastritis and vitamin B_{12} deficiency in subjects with corpus atrophy, it can be estimated that there are thousands of elderly people in Finland (total population approximately 5 millions) who have or who are at high risk for the deficiency of vitamin B_{12} caused by the atrophic gastritis, in the majority of whom (80% at least) the atrophic gastritis is initiated by *H. pylori* infection, and who still have an undiagnosed ongoing *H. pylori* infection.

H. pylori test alone is not enough to diagnose the patients at risk for gastric cancer.

Breath test and antigen stool tests are commonly used to diagnose *H. pylori* infection in the general practice. Both tests

Table 1. **Diagnostic algorithms based on biomarker tests (GastroPanel) from a blood sample**

Biomarker test result	Interpretation and conclusions
Normal (healthy) stomach	Risk of cancer and peptic ulcer is practically nil (except in users of NSAID and aspirin). Consider other examinations first than gastroscopy. Colonoscopy, abdominal ultrasound and other examinations may be more beneficial than gastroscopy. PPI may be helpful in cases with reflux symptoms, or if gastrin-17 is very low – stomach is hyperchlorhydric – there is a risk of acid related damages in esophagus and cardia in patients with gastroesophageal reflux [34].
Non-atrophic *H. pylori* gastritis	Risk of gastric cancer is low whereas the risk of peptic ulcer may be considerable. Gastroscopy may be of low diagnostic help. Consider the treatment of *H. pylori* (prevention of gastric cancer and peptic ulcer).
Atrophic gastritis irrespective whether *H. pylori* (+) or (-)	Cancer risk is considerable. Careful diagnostic gastroscopy is mandatory to diagnose a possible cancer or precancer lesion (occur in up to 5% of cases). Treatment of *H. pylori*, if present, is recommended (cancer prevention). Test serum B_{12} if corpus is atrophic. No need for PPI, if corpus mucosa is atrophic (endogenous acid secretion is low). No risk of peptic ulcer if corpus mucosa is atrophic (endogenous acid secretion is low) but the ulcer risk, the risk of gastric ulcer in particular is high, if the atrophic gastritis is limited to antrum alone.

are reliable in cases with florid and extensive infection but are handicapped in cases with severely atrophic and hypochlorhydric stomach, as often is the case in patients with high cancer risk [11-14]. These direct tests only address an answer as to whether the patient has an *H. pylori* infection – nothing else. They are, however, unable to answer the question whether the patient has atrophic gastritis, and whether the patient is, therefore, at risk for gastric neoplasia. These direct tests, also including endoscopic biopsy urease test, can neither provide reliable evidence of whether the gastric mucosa is certainly normal and healthy. Particularly in the elderly, the negative breath test does not exclude the possibility that the stomach is severely sick, or that the patient would not have, for example, a severe *H. pylori* – negative atrophic gastritis. The direct tests often give false negative results particularly in patients with atrophic gastritis, hypochlorhydria and intestinal metaplasia, obviously due to reduction in *H. pylori* load in the gastric mucosa. This low colonization of bacteria is also the cause for common false negative results by breath test, or by antigen stool test, in subjects under the PPI treatment [15-18]. In fact, if one relies only on direct *H. pylori* tests, the risk of false negative test result, and the consideration that the patients has a healthy stomach, tends to be highest just among the subjects with highest cancer risk.

How to interpret the blood biomarker tests?

My personal view and advices to interpret the biomarker tests are presented in *Tab. 1* [see also refs 18-34]. It gives some personal suggestions only. It should be noticed, however, that all patients with severe and alarming symptoms (severe pain, bleedings, weight loss, etc.) must always be referred to immediate gastroscopy and endoscopic biopsies, without any prior tests. The suggestions given are advisable and valid only with tests of high quality that include assays of *H. pylori*, pepsinogens I and II, as well as gastrin-17 (GastroPanel).

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Antioxidant mechanism of hepatoprotection by ursodeoxycholic acid in experimental alcoholic steatohepatitis

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Abstract

Purpose: The aim of this study was to evaluate the role of an antioxidant factor in the hepatoprotective effect of ursodeoxycholic acid (UDCA) in rat alcoholic steatohepatitis.

Material and methods: The effects of UDCA (40 mg/kg, i.g., 30 days) were studied using rats fed on a high-fat diet (52% calories as fat) and administered with ethanol via intragastric intubation (4 g/kg daily, 30 days) .

Results: The livers of ethanol-treated animals were characterized by fatty dystrophy. The relative liver weight and the square of the sudanophylic area as well as the liver triglyceride content and the activity of the serum marker enzymes, aspartate aminotransferase and γ-glutamyltransferase, were significantly increased. Elevated superoxide dismutase activity as well as increased contents of lipid peroxidation products (hydroxyalkenals, malone dialdehyde, etc.) and lucigenin-enhanced microsomal chemiluminescence were observed in the liver of ethanol-treated rats and the liver reduced glutathione content was decreased. An increase in monoenoic fatty acids, a decrease of the n-6 acid family and an enhancement of microsomal membrane viscosity were found in the liver of these animals. An elevation of the total cytochrome P-450 content and the activity of amidopyrine-N-demethylase were shown in liver microsomes of the ethanol-treated group. The UDCA treatment improved the liver morphology, decreased serum marker enzyme activities, liver triglyceride content and normalized all the indices of oxidative stress. UDCA lowered the viscosity of the microsomal membrane, as assessed by both the fluorescence probe techniques and the saturated/unsaturated fatty acid ratio. The microsomal cytochrome P-450 content and amidopyrine-N-demethylase activity were normalized in UDCA-treated rats.

Conclusions: We can conclude that the hepatoprotective effect of UDCA stipulated by its antioxidant properties is indeed the factor enabling UDCA to control metabolic processes by changing the properties of liver membranes and membranous proteins.

Key words: alcoholic steatohepatitis, ursodeoxycholic acid, liver membranes, oxidative stress.

Introduction

Hepatoprotective properties of ursodeoxycholic acid (UDCA) in alcoholic liver injury have been described quite recently [1,2]. Throughout several decades UDCA has been of considerable clinical use to treat cholestatic liver injuries. The mechanism of the UDCA hepatoprotective effect has been interpreted by the fact that, as a hydrophilic bile acid, it displaces toxic bile acids from the membranes, thus protecting the latter from destruction [3]. However, the protective effect of UDCA on liver alcoholic steatohepatitis cannot be explained in the context of the above hypothesis since this pathology does not involve cholestatic phenomena and, as a consequence, membrane infiltration by hydrophobic bile acids. Some authors explained the UDCA hepatoprotective effect in alcoholic liver injury by its beneficial influence on mitochondrial oxidation [4], improvement of plasma membrane physical properties [1] and recovery of liver prostaglandin level [2].

Numerous experimental and clinical findings suggest that free radical mechanisms contribute considerably to ethanol-induced liver injury [5-7]. The hypothesis for the role of oxidative stress in the pathogenesis of alcoholic liver injury has become generally recognized and serves as a basis for applying antioxidants in treatment of this pathology. However, treatment of alcoholic liver injury by antioxidant drugs has not received a

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wide recognition. For example, clinical estimates of vitamin E efficacy in treatment and prevention of alcoholic liver injury have been ambiguous [8]. At the same time, clinical application of another antioxidant, silymarin, in this pathology seems to be promising [9]. Some authors believe the use of metal chelators, neutralizing the prooxidant effect of iron, to hold much promise as a means for treating alcoholic liver injury [7]. The hepatoprotective effects of prostaglandin E2 and polyunsaturated phosphatidylcholine were also partially achieved by antioxidant mechanisms [10,11].

In our laboratory the antioxidant effect of UDCA *in vivo* was first found when using rats with oxidative stress induced by 1-Gy single γ-irradiation [12]. UDCA lowered the generation of reactive oxygen species and the content of lipid peroxidation end-products in the liver. In this context, it was interesting to assess the antioxidant potential of this compound in alcoholic liver steatohepatitis which, as literature data show, is accompanied by activation of free radical generation [6].

Material and methods

Male albino Wistar rats with an initial body weight ranging from 190 to 210 g were used. The rats from all the groups were fed on a high-fat diet (HFD) *ad libitum* for 30 days. The diet contained the following components (in %): casein, 21.0; starch, 41.5; fat (lard and sunflower oil, 1:1 wt/wt), 26.0; cellulose, 6.5; and vitamin and mineral mix, 5.0 [13]. The diet provided 52% of metabolizable energy as fats. For 30 days, two groups of the animals were treated with ethanol via a gastric tube (4 g/kg b.w., daily). The animals from the second ethanol-treated group were administered with a UDCA aqueous suspension (40 mg/kg b.w., daily). Both groups treated with ethanol (the ethanol group) and ethanol combined with UDCA (the ethanol + UDCA group) were simultaneously fed on a HFD throughout the experiment. The control group fed on a HFD (the HFD group) for 30 days received intragastrically isocaloric amount of sucrose. Each group was of 8 animals.

The rats were decapitated under pentobarbital anaesthesia after 12-h starving. Blood serum was obtained by centrifugation at 3000 g. The liver microsomal fraction was isolated by differential centrifugation at 105000 g using a VAC-602 centrifuge (Janetzki, Germany).

For histological studies, liver samples were fixed in Bouin solution. Histological sections were prepared and stained with hematoxylin and eosin. For histochemical assessment of neutral lipids, liver samples were frozen in liquid nitrogen and cryostat sections were stained with Sudan black B. The sudanophilic areas were measured with a BIOSCAN-NT image computer analyzer (Minsk, Belarus).

The activities of the serum marker enzymes, alanine aminotransferase (AlAT) and aspartate aminotransferase (AsAT), were measured by using commercially available kits (Lachema, Czech Republic).

Lipids were extracted with a chlorophorm : methanol mixture (2:1, v/v) [14]. Neutral lipids were separated by thin-layer chromatography into classes [15]. Individual spots corresponding to lipid fractions (triglycerides, phospholipids) were scraped and extracted by methanol. Triglycerides were measured by routine methods using commercial kits from Lachema (Brno, Czech Republic) according to manufacturer instructions. Phospholipids were used for gas-chromatographic determination of fatty acid pattern. Phospholipid fatty acids were methylated with 1.75 M sulphuric acid in absolute methanol at 75°C and separated with a 3700 model gas chromatograph (Moscow, Russia) equipped with a flame ionisation detector and a 1.5x3 mm glass column packed with 10% DEGS on Inerton-Super 100/120 mesh. The temperatures were as follows: column, 197°C; injector, 250°C; detector, 250°C. The flow rates were the following: carrier gas (helium), 30 ml/min; hydrogen, 30 ml/min; air, 300 ml/min. Fatty acid peaks were identified by comparison of the retention time with authentic standards (U.S. Biochemical Corporation, USA).

The activity of superoxide dismutase was measured by the modified method with nitrotetrazolium blue [16]. The method was based on competition of superoxide dismutase and nitrotetrazolium blue for the superoxide ion produced due to interaction of NADPH and phenazine metasulphate. The dinitrophenyl hydrazone mixture was separated in polar and non-polar zones [17]. The extract containing dinitrophenyl hydrazones of aldehydes was separated by double TLC development on 20x20 cm silica gel G-60 plates of 0.25 mm thickness. Dichloromethane followed by benzene were used as mobile phases. Non-polar hydrazones were found in three zones: 4-hydroxyalkenals (zone 1), ozazones (zone 2), alkenals, alkanals and ketones (zone 3). They were identified by the corresponding standards. Individual fractions were scraped, extracted by methanol and determined spectrophotometrically at 365 nm.

NADPH-dependent liver microsomal chemiluminescence was conducted by the use of the chemiluminogenic probes, luminol and lucigenin [18]. The incubation mixture, containing 0.1 M phosphate buffer (pH 7.4), 0.1 μM iron sulphate (II), $6x10^{-4}$ mM luminol and lucigenin, 0.1 nM NADPH and microsomal suspension (5 mg protein), was placed in a cell. The reaction was started by addition of microsomal protein. Chemiluminescence was recorded with a BLM-100 bioluminometer (Krasnoyarsk, Russia) for 4 min.

Lipid peroxidation was assessed by the malone dialdehyde (MDA) level as determined by a thiobarbituric acid method [19]. The molar extinction coefficient of $1.56x10^5$ mol^{-1} cm^{-1} was used to calculate MDA concentration. Trichloroacetic acid was added to control samples before NADPH addition.

Reduced free thiols, among which reduced glutathione (GSH) was more abundant, were measured by the modified method of Ellman [20]. The absorption was recorded at 412 nm with a Specol-211 spectrophotometer (Carl Zeiss, Germany).

The content of cytochrome P-450 in the liver microsomal fraction was determined by the method of Omura and Sato [21]. Amydopyrine-N-demethylase activity was measured according to Klinger and Muller [22]. The incubation mixture (1 ml) contained 100 mM Tris-HCl buffer, (pH 7.4) and 5 mM $MgCl_2$, 8 mM amydopyrine and 2 mg microsomal protein. The reaction was started by addition of 3 mM NADPH, and after 5-min incubation at 370°C for 45 min, absorption was measured at 412 nm with a Specol 221 spectrophotometer and compared to control. Formaldehyde was determined applying the calibration curves constructed by the use of standard solutions.

Figure 1. **Lipid accumulation in the liver. Staining with Sudan black; magnification 10 x. A – Control; B – Ethanol; C – Ethanol + UDCA**

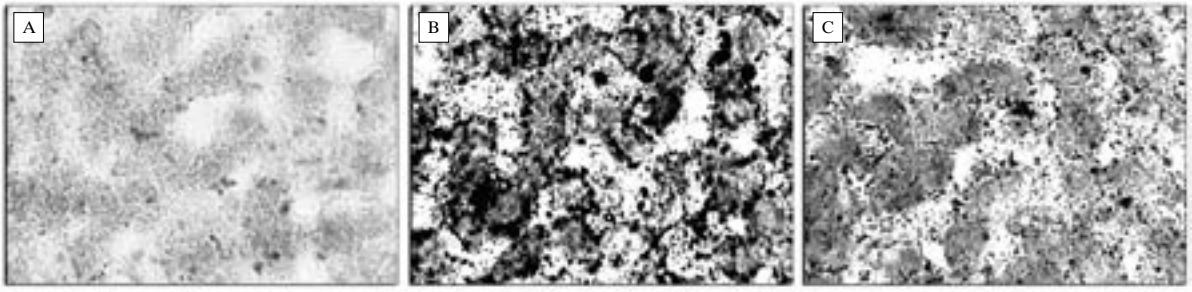

Table 1. **Morphologic characteristics of the liver and serum marker transferases (μmol/mg protein per 1 min) and liver triglyceride content in experimental rats**

Parameter	Control	Ethanol	Ethanol + UDCA
Liver relative weight (g/100 g body weight)	3.12±0.29	4.10±0.26[a]	3.29±0.21
Relative square of sudanophylic area (% to slide square)	0.026±0.0078	0.092±0.040[a]	0.025±0,003[b]
AsAT	1.17±0.088	1.63±0.112[a]	1.03±0.099[b]
AlAT	0.87±0.055	0.98±0.082	0.77±0.070
GGT	2.16±0.38	7.14±0.83[a]	3.89±0.46[ab]
Triglyceride	1618±110	4825±384[a]	1881±57[b]

Values are means ±SEM for eight rats; [a] – P<0.05 compared with the control group; [b] – P<0.05 compared with the ethanol-treated group

The fluorescence spectra were recorded on a LOMO--SDL-2 spectrofluorimeter (Russia) equipped with a xenon lamp as a fluorescence source. The fluorescence intensities (I) of pyrene monomer and eximers were measured at height of fluorescence band at 373 and 470 nm, respectively. A TRS-2850 standard wolfram lamp was used to calibrate the spectrofluorimeter. The membrane suspensions (5 μl), containing 0.1 ml protein/ml, were titrated with 5 μl portions of pyrene solution in ethanol with intensive stirring. The fluorescence spectra were recorded after 2 min incubation at 250°C with the regular portion of the probe. The maximal volume of pyrene solution added was 50 μl.

The data were processed statistically using two-tailed Student's t-test. The minimal level of significance was set at $p<0.05$.

Results

The long-term treatment of rats fed on the HFD simultaneously with ethanol provoked steatohepatitis which was characterized morphologically by lipid accumulation in hepatocytes and lymphocyte infiltration in the parenchyma. The Sudan black-stained liver preparations showed a diffuse distribution of lipid inclusions, with the lipid vacuoles enlarging to medium sizes, whereas slides from control animals demonstrated very fine, almost indistinguishable, lipid inclusions (*Fig. 1a*, *b*). The morphometric findings suggested an increased relative area of sudanophilic regions on alcohol-treated rat liver preparations (3.5-fold, *Tab. 1*).

The biochemical parameters indicated the presence of alcoholic liver injury in rats treated with ethanol. The activities of the serum marker enzymes, γ-glutamyl transferase and AsAT, were significantly elevated, with AlAT activity remaining essentially unchanged (*Tab. 1*). The content of liver triglycerides was raised 3-fold, which confirms enhanced accumulation of neutral lipids in the liver of the ethanol group. The average liver weight in these animals was also considerably increased.

The administration of UDCA (40 mg/kg, i.g., 56 days) to alcohol-treated animals fed on the HFD essentially normalized the morphologic picture of the liver. Along with the normal liver structure, the animals from this group had some diffuse lymphocyte infiltrations in the parenchyma. However, the sizes and the number of lipid vacuoles were much smaller compared to those in rats treated with ethanol alone (*Fig.1b*). The morphometric evaluation of liver slices stained with Sudan black indicated a pronounced decrease of neutral lipid accumulation in the liver after the application of UDCA (*Tab. 1*).

The UDCA treatment normalized the activity of serum AsAT and significantly decreased that of γ-glutamyl transferase. The content of liver triglycerides reduced virtually to the control values (*Tab. 1*).

The long-term ethanol treatment increased the viscosity of liver microsomal membranes as assessed from the fluorescence intensity ratio of the pyrene monomer to eximer fractions (I372/I470).

In animals with alcoholic steatohepatitis UDCA considerably decreased this parameter (*Tab. 2*). However, the membrane polarity, as shown by the I372/I395 ratio, was unaffected in all the experimental groups.

The increased concentrations of palmitic, palmitoleic and eicosatrienic acids and the diminished level of arachidonic acid were found in liver microsomal phospholipids of animals with alcoholic steatohepatitis (*Tab. 3*). As opposed to the HDF

***Table 2*. The fluorescence intensity of the fluorescent probe pyrene (I_{372}), bound to the liver microsomal membrane, ratio of oscillation band intensities at 372 and 395 nm (I_{372}/ I_{395}), and ratio of the intensities at 372 and 470 nm (I_{372}/ I_{470}) in experimental rats**

Parameter	Control	Ethanol	Ethanol + UDCA
I_{372}	0.84 ± 0.03	1.19 ± 0.05[a]	0.83 ± 0.04[b]
I_{372}/ I_{395}	1.16 ± 0.06	1.15 ± 0.04	1.13 ± 0.04
I_{372}/ I_{470}	4.16 ± 0.12	5.38 ± 0.19[a]	2.11 ± 0.06[ab]

Values are means ± SEM for six rats; [a] – $p<0.05$ compared with the control group; [b] – $p<0.05$ compared with the ethanol-treated group

***Table 3*. Effect of ethanol consumption and UDCA treatment on fatty acid composition (% to total fatty acids) of microsomal phospholipids from the liver of rats pair fed the high-fat diet**

Fatty acid	Control	Ethanol	Ethanol + UDCA
C 16:0	12.7±0.78	14.7±0.56[a]	12.8±0.39[b]
C 16:1 (n-7)	3.5 ±. 27	6.3±0.26[a]	4.3±0.19[b]
C 18:0	20.8±0.75	20.1±0.82	13.8±0.78[ab]
C 18:1 (n-3)	16.8±0.66	18.4±0.71	21.4±0.63[ab]
C 18:2 (n-6)	9.5±0.79	7.6±0.68	13.2±0.73[ab]
C 20:3 (n-3)	0.8±0.07	1.3±0.11[a]	0.4±0.01[ab]
C 20:4 (n-6)	24.3±1.83	17.3±1.56[a]	20.1±2.97
C 20:5 (n-3)	1.1±0.11	1.0±0.12	1.1±0.08
C 22:2 (n-9)	2.5±0.19	2.6±0.23	2.2±0.23
C 22:3 (n-9)	1.4±0.12	1.8±0.17	1.4±0.10
C 22:4 (n-9)	2.4±0.24	2.5±0.25	2.0±0.18
C 22:5 (n-6)	3.0±0.28	2.1±0.22	4.5±0.42[ab]
C 22:6 (n-3)	3.6±0.31	4.0±0.25	3.8±0.33
Saturated/ unsaturated acids	0.51±0.01	0.59±0.02[a]	0.38±0.03[b]

Values are means ± SEM for six rats; [a] – $p<0.05$ compared with the control group; [b] – $p<0.05$ compared with the ethanol-treated group

group, rats administered with ethanol + UDCA had considerably raised percentage of oleic acid, linoleic acid and its derivatives as well as docosapentaenic acid, the levels of stearic acids and group n-9 fatty acids being reduced. The concentrations of palmitic and palmitoleic acids in the ethanol + UDCA group were lower compared to the findings for the group treated with ethanol alone. The ratio of saturated/unsaturated fatty acids, which reflected the viscosity of cell membranes [23], was raised in the ethanol-treated group and decreased after the UDCA administration.

The prooxidant effect of the long-term ethanol administration manifested itself in activation of free radical generation as demonstrated by chemiluminescence intensity of liver microsomes enhanced by luminol and lucigenin, increased superoxide dismutase activity, and lipid peroxidation end-product contents (MDA and polar carbonyls) as well as in a decrease of reduced free thiols (*Tab. 4*). The treatment with ethanol increased nearly two-fold the total cytochrome P-450 content and amidopyrine-N-demethylase activity in liver microsomes.

Our findings show that alongside with the pronounced hepatoprotective effect, the UDCA administration reduced the production of reactive oxygen forms in the liver, the content of lipid peroxidation carbonyl-containing products (alkenals, alkanals, ketones, oxyalkenals and MDA), and the activities of antioxidant defence enzymes (superoxide dismutase) and increased the concentraton of reduced free thiols (*Tab. 4*). Moreover, UDCA normalized liver microsomal cytochrome P-450 level and the activity of amidopyrine-N-demethylase, the end acceptor of the cytochrome P-450-dependent electron transport chain.

***Table 4*. Parameters characterizing oxidative stress and lipid peroxidation in the liver and cytochrome P-450-related indices in liver microsmes of experimental rats**

Parameter	Control	Ethanol	Ethanol + UDCA
NADPH-induced chemiluminescence enhanced by lucigenin, c.p.m./mg microsomal protein per 1 min x 10^6	1.54±0.34	2.92±0.55[a]	1.79±0.31[b]
NADPH-induced chemiluminescence enhanced by luminol, c.p.m./mg microsomal protein per 1 min x 10^3	2.13±0.33	16.65±2.17[a]	2.96±0.49[b]
MDA content, nmol/mg liver protein	0.76±0.049	1.55±0.099[a]	0.64±0.104[b]
SOD activity, μmol/mg cytosolic protein per 1 min	1.72±0.341	2.48±0.358[a]	1.14±0.307[b]
Alkanals, alkenals and ketones content, nmol/mg liver protein	175±21	295±32[a]	204±18[b]
Hydroxyalkenals content, nmol/mg liver protein	99±11	159±16[a]	112±12[b]
Reduced glutathione content, μmol/mg liver protein	5.2±0.31	3.0±0.19[a]	5,5±0.25[b]
Total cytochrome P-450 content, nmol/mg microsomal protein	0.76±0.050	1.550±0.099[a]	0.692±0.104[b]
Amydopyrine-N-demethylase activity, nmol/mg microsomal protein per 1 min	4.21±0.48	8.61±0.73[a]	5.45±0.68[b]

Values are means ± SEM for eight rats; [a] – $p<0.05$ compared with the control group; [b] – $p<0.05$ compared with the ethanol-treated group

Discussion

The pronounced hepatoprotective effect of UDCA in alcoholic liver injury, which we confirmed in the present work, seems to be a multifactor process. In a few studies on this problem, the defence UDCA-governed mechanisms involve improvement of mitochondrial structure and ATP production-related mitochondrial oxidation [4], as well as stabilization of hepatocyte plasma membrane physical properties [1] and recovery of the level of hepatoprotective prostaglandins E [2] in the liver of alcohol-treated rats. Many authors attribute the UDCA defence of liver mitochondria to possible antioxidant properties of this compound [24,25] and stabilization of liver mitochondrial membranes.

The antioxidant effect of UDCA has been studied rather intensively during the last decade. Most frequently, experiments *in vitro* provided a negative answer to this problem. For example, investigators did not find any changes in superoxide anion production by either monocytes of UDCA-treated patients with cholestatic diseases or by cells taken from healthy individuals and preincubated with UDCA [26]. UDCA did not affect the MDA production by macrophage culture and the generation of reactive oxygen forms by rat liver mitochondria, but it prevented a prooxidant effect of hydrophobic bile acids [27,28]. Furthermore, UDCA prevented reduced glutathione depletion in cultivated hepatocytes treated with hydrogen peroxide and cadmium [29]. Thus, the experiments *in vitro* did not confirm with certainty the antioxidant effect of UDCA. In contrast, in studies *in vivo* UDCA acted as a rather effective antioxidant. In rats with alcoholic liver injury, UDCA reduced the content of lipid peroxidation end-products both in the whole liver [30] and in its subcellular fractions, mitochondria [1] and microsomes [31]. UDCA inhibited lipid peroxidation products in the liver of rats with cholestasis provoked by a bile duct ligation [32]. In the liver of rats with γ-irradiation-induced oxidative stress, UDCA normalized the activity of superoxide dismutase, the contents of superoxide radical and lipid peroxidation carbonyl-containing products, diminished chemiluminescence enhanced by luminol and lucigenin and prevented a decrease of reduced glutathione [12]. The distinctions in *in vivo* and *in vitro* results may indicate that the UDCA antioxidant effect may be accomplished via its metabolites.

Moreover, the administration of UDCA normalized the level of liver cytochrome P-450 and the activities of the matched microsomal reactions [31]. Since cytochrome P-450 is the primary source of liver free oxygen radicals in alcohol intoxication, we have every reason to believe that at least one of the mechanisms of the UDCA antioxidant action may be mediated through its effect on the cytochrome P-450 system which, in turn, is involved in bile acid biotransformation [33,34].

The mechanisms of UDCA membranotropic effect, which have been well-studied in cholestatic liver injury [3], do not apply to liver diseases which occur with cholestasis, particularly to alcoholic steatohepatitis. Since in cholestatic diseases the key mechanism of the UDCA therapeutic effect is a replacement of toxic hydrophobic bile acids in membranous structures, alternative mechanisms should be considered for alcoholic liver injuries. We should, however, specify that the UDCA-induced enhanced membrane fluidity may partly be explained in terms of a possible competition with cholesterol for membranous binding sites [35,36]. However, we are inclined to believe that the changes in liver membrane physical properties observed under long-term UDCA administration are related to the increased ratio of unsaturated/saturated fatty acids of the phospholipid microsomal membrane, which in its turn can be accounted for by the antioxidant effect of UDCA preventing membranous fatty acid peroxidation.

The literature data consideration allows us to conclude [37] that hepatoprotective medicinal preparations for treatment of alcoholic liver injuries must at least: a) possess antioxidant properties; b) stabilize cell membranes; c) inhibit microsomal cytochrome P-450-dependent system activity as the primary source of oxygen free radicals in alcohol intoxication [6]; d) have anti-inflammatory properties. From this classification, a substance with the above properties may be considered an ideal hepatoprotector. On the basis of the above data UDCA may be assigned to the category of such "ideal" hepatoprotectors: it possesses antioxidant activity, inhibits the activity of the cytochrome P-450-dependent system of xenobiotic metabolism activated by long-term ethanol consumption; stabilizes physical and chemical properties of the membrane. Moreover, UDCA inhibits production of the principal anti-inflammatory cytokine, TNFα [38]. We can conclude that the hepatoprotective effects of UDCA in alcoholic steatohepatitisis are mediated, at least in part, by its antioxidant actions which are probably linked to an inhibition of cytochrome P-450-related free radical generation. The antioxidant effects of UDCA lead to improvement of liver membrane physical properties and functions which in turn stipulate protection of the liver against the damaging effects of ethanol and oxidative stress accompanying alcoholic liver injury.

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TGF-β1 down-regulates ICAM-1 expression and enhances liver metastasis of pancreatic cancer

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Abstract

Purpose: In order to study the regulation of adhesion-molecule expression by cytokines, we have investigated the effect of transforming growth factor-β1 (TGF-β1) on the expression of intercellular adhesion molecule-1 (ICAM-1) in human pancreatic cancer cell lines.

Material and methods: By using three pancreatic cancer cell lines, SW1990, CAPAN-2 and PANC-1, the effect of TGF-β1 on expression of ICAM-1, cancer cell immunogenicity and liver metastasis were investigated.

Results: Cell surface ICAM-1 expression by ELISA on three cell lines were all reduced significantly by following incubation with various concentrations of TGF-β1 and down-regulation of ICAM-1 expression was also observed at the mRNA level. Corresponding to the down expression of ICAM-1, the adhesion of peripheral blood mononuclear lymphocytes (PBMLs) to cancer cells and cancer cell cytotoxicity during co-culture with PBMLs were remarkably decreased by treatment with TGF-β1. Furthermore, enhanced liver metastatic potential by *in vivo* splenic injection was observed in CAPAN-2 cells pre-treated with TGF-β1.

Conclusions: Since decreased expression of ICAM-1 has been known to contribute to cancer cell escape from immunologic recognition and cytotoxicity by effector cells, the present results indicate that unknown function of TGF-β1 in the tumor progression and metastasis of pancreatic cancer.

Key words: pancreatic cancer, liver metastasis, ICAM-1, TGF-β1, immunosuppression.

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Introduction

The progression and metastasis of malignant tumor has been known to be mediated by various factors at microenvironment. Once tumor cells escape from the recognition by immunosurveillance system, it may acquire further metastatic properties. Cellular adhesion mediated by various membrane-associated adhesion molecules are essential for the proper function of immunologic processes. One of these adhesion molecules, intercellular adhesion molecule-1 (ICAM-1, CD54) is known to play an important role in the interaction between tumor cells and host cytotoxic effector cells [1].

ICAM-1 is a member of the immunoglobulin superfamily and a ligand for lymphocytes function-associated antigen-1 (LFA-1), which is expressed on T cells. Natural killer (NK) cells and lymphokine-activated killer (LAK) cells lead the recognition and subsequent lysis of appropriate target cells through ICAM-1/LFA-1 system, and cytotoxic T lymphocytes (CTLs) also require ICAM-1 expression as a co-stimulating accessory molecule in the activation of their immune response through T cell receptor [2,3]. The induction of ICAM-1 expression is regulated by several cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), interleukin-2 (IL-2) and interleukin-6 (IL-6) as well as lipopolysaccharide (LPS), and this up-regulation may be benefit for cancer cell killing [4]. However, down-regulation of ICAM-1 expression and regulation of sICAM-1 level still remain to be unknown.

Transforming growth factor-β1 (TGF-β1) inhibits potently tumor growth of normal and tumor epithelial cells *in vitro* and can exert multifunctional biologic effects in differentiation, inflammation, tissue repair and extracellular matrix remodelling besides cell proliferation [5]. TGF-β1 also stimulates cancer cell migration, production of proteolytic enzyme degradating extracellular matrix and suppression of immune response which play important roles in the tumor progression and metastasis [6]. Furthermore, stimulatory and inhibitory effects on cell adhesion by regulating the expression of cell surface adhesion molecules might be involved in the key potential function of TGF-β1.

In this study, we studied the relationship between metastatic potential and ICAM-1 expression in pancreatic cancer cells, and also investigated the modulating effect of TGF-β1 in ICAM-1 expression, cancer cell immunogenicity and liver metastasis.

Material and methods

Cell Lines and Culture

Human pancreatic cancer cell lines, SW1990, CAPAN-2 and PANC-1 were used in this study and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml of penicillin, 100 μg/ml of streptomycin and 2 mM glutamine at 37°C in 5% CO_2 humidified atmosphere.

Flow Cytometric Analysis

Flow cytometric analysis was performed according to the method previously described [7]. All cells were prepared as cell suspension, and incubated with anti-human ICAM-1 antibody (84H10, Immunotech, France). Cells were washed twice and incubated with fluorescein isothio-cyanate-conjugated goat anti-mouse immunoglobulin (Tago, Buringame, CA, USA), and were analysed using EPICS ELITE ESP flow-cytometer (Coulter Co., USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

ICAM-1 expression on cancer cell surface was determined by ELISA system according to the method previously described [8]. Cells were cultured in 96-well microplates over a 48 h period till reaching to confluent and treated by recombinant human TGF-β1 (Austral Biologicals, CA, USA) at various concentrations (0.8, 4.0, 20.0 ng/ml) and 2.0, 10.0 and 50.0 ng/ml of recombinant human TNF-α (Genzyme, MA, USA) at 37°C for 24 h. After cells were washed with PBS, they were fixed with 0.25% glutaraldehyde and incubated with anti-ICAM-1 antibody after blocking with 100 mM glycine, 1% bovine serum albumin (BSA) in PBS. The quantitative expression of ICAM-1 was determined with horseradish peroxidase-conjugated second antibodies.

Northern Blot Analysis

Cells were spread on to 10 cm plastic dish and incubated over night, then treated with 20 ng of TGF-β1 or 50 ng of TNF-α for 24 h. Total RNA was extracted by Acid Guanidium-Phenol-Chloroform method and Northern blot hybridization was performed as previously described [9]. Briefly, twenty-five mg of total RNA was electrophoresed in 1% denaturing agarose gel and blotted on to nylon membrane. The blot was hybridized to ICAM-1 oligonucleotide probe (#ON398, Oncogene Science Inc., Cambridge, MA) labeled with 32P using endlabeling method (Megalabel 5'-endlabeling kit, Takara, Shiga, Japan). Excess probes were then washed out. Autoradiography was subsequently performed, and the intensity of the band displaying specific ICAM-1 expression was measured by BAS 2000 system (Fuji Film, Tokyo, Japan).

Lymphocytes adhesion assay to cancer cells

Human peripheral blood mononuclear lymphocytes (PBMLs) were prepared from healthy volunteers by Ficoll-Hypaque density gradient centrifugation. Mono-Poly Resolving Medium was added to plastic tubes and fresh anti-coagulated blood were added onto the medium. After centrifugation at 1500 rpm for 30 min and elimination of supernatant, PBMLs were collected and resuspended in DMEM with 10% FCS. Cancer cells (2.5×10^5/well) were incubated in DMEM with 10% FCS alone or 10% FCS and TGF-β1 (20 ng/ml) or anti-ICAM-1 neutralizing antibody (2 μg/ml) for 48 h in 96-well microplates. After washing plates with PBS, PBMLs (2.5×10^6/well) suspended in serum-free medium were allowed to attach to cancer cells for 1 h at 37°C. The binding of PBMLs was quantified by colorimeric assay with MTT [10] using a MTP-120 Microplate reader at 550 nm. The percentage of total PBMLs that adhered to cancer cells was calculated as % adhesion = (OD of experimental wells – OD of cancer cell wells)/OD of total PBML wells×100.

Cancer cell cytotoxicity assay

The cell cytotoxicity assay was performed using a Cytotox 96 Non Radioactive Cytotoxicity Assay Kit (Promega Co., Madison, WI), that measured lactate dehydrogenase (LDH). After cancer cells were pretreated with or without TGF-β1 (20 ng/ml) or anti-ICAM-1 neutralizing antibody (2 μg/ml) for 48 h prior to assay, approximately 1×10^4/well cancer cells were placed in 96-well plate, and 1×10^5/well PBMLs were added and incubated at 37°C for 18 h. Absorbance related to released LDH level in the medium was measured with Microplate reader at 492 nm. The percentage of total cancer cells that underwent lysis was calculated as % cytotoxicty=(experimental – PBML spontaneous – cancer spontaneous)/(cancer maximum – cancer spontaneous)×100.

In vivo liver metastatic assay

Cancer cells were incubated in 10% FCS-DMEM with or without 20 ng/ml TGF-β1 for 48 h. The cells were suspended to a final concentration of 5×10^5/0.1 ml PBS and injected into the spleen of 6-week-old female Balb/c nude mice under ether anesthesia. After injection, the spleen was extracted and mice were sacrificed after 5 weeks to measure the number of metastatic tumor.

Statistical analysis

Results were expressed as the mean ±SD. Student's t-test was used for statistical analysis and P values less than 0.05 were considered to indicate statistical significance.

Results

ICAM-1 Expression on Pancreatic Cancer Cells

The percentage of ICAM-1 positively expressing cells in SW1990, CAPAN-2 and PANC-1 were 27.1, 73.2 and 94.1, respectively by analysis with flow cytometry. While the expression of HLA class I antigen on these three cell lines was also analysed with monoclonal antibody B9.12.1 against this antigen (Immunotech), there was no difference between these cell lines with high percentage of expression (99.7, 100 and 99.6, respectively) (*Fig. 1*).

Cell surface ICAM-1 expression by ELISA

Subsequently, the expression of ICAM-1 were determined quantitatively by ELISA, and SW1990 cells demonstrated signifi-

Figure 1. **HLA class I antigen and ICAM-1 expression on pancreatic cancer cell lines by flow cytometric analysis**

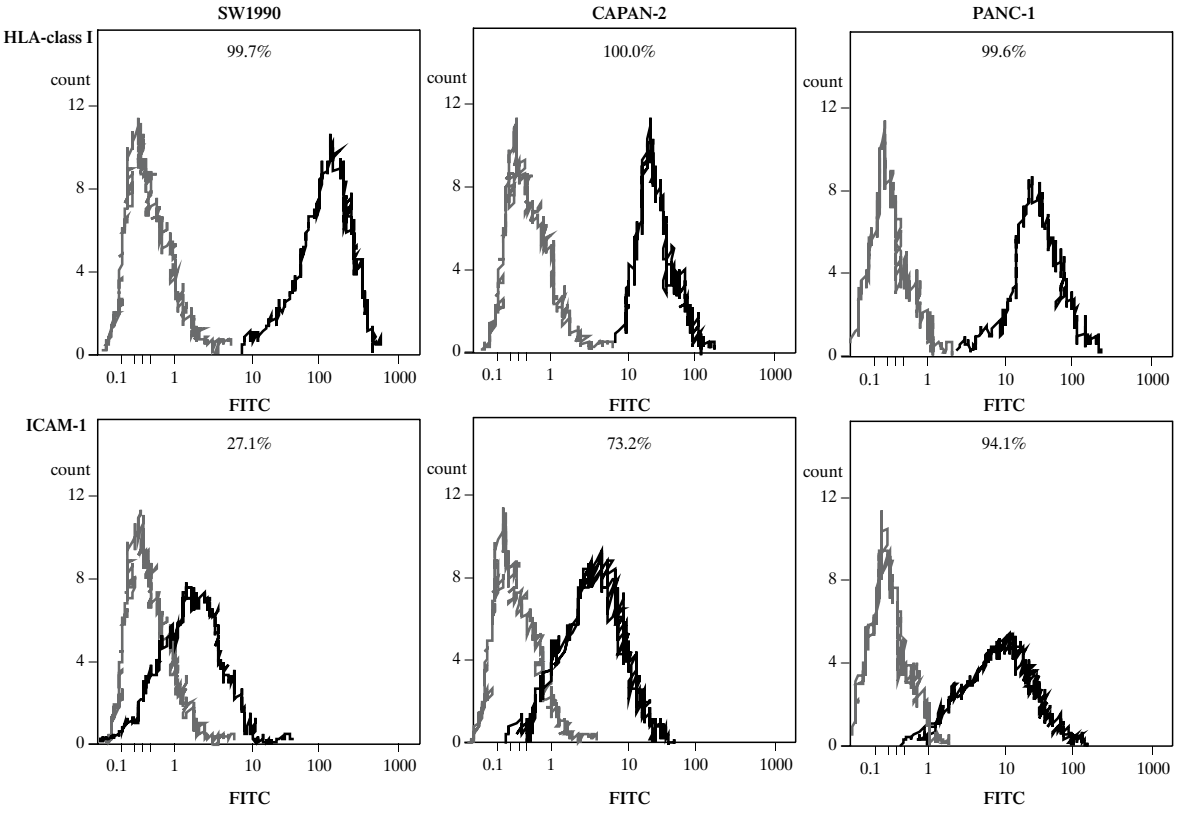
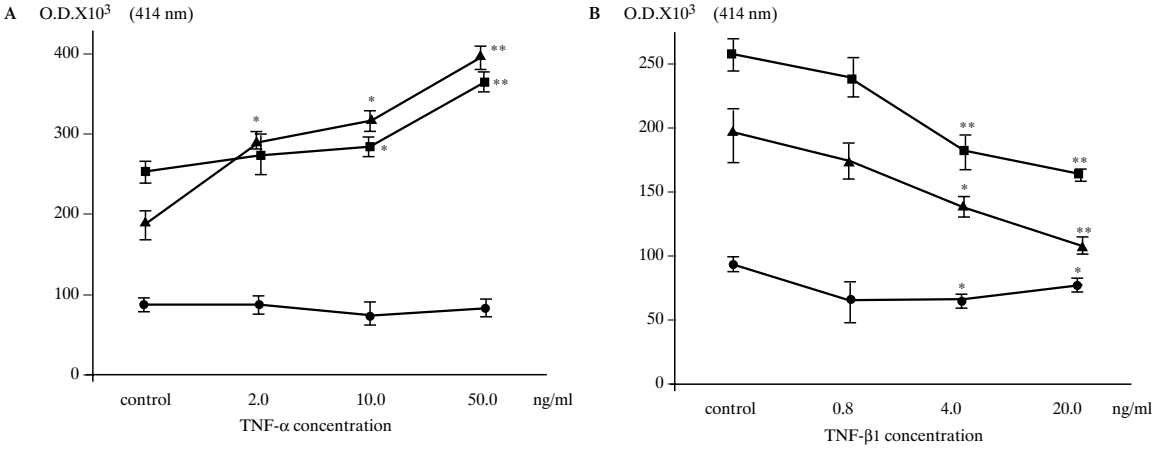


Figure 2. **Modulation of ICAM-1 expression by TNF-α (A) and TGF-β1 (B) on pancreatic cancer cells. The ICAM-1 expression on cell surface of SW1990 (•), CAPAN-2 (▲) and PANC-1 (■) were determined by ELISA system. (*P<0.05, **P<0.01)**



cantly lower expression of ICAM-1 compared with CAPAN-2 and PANC-1 consistently with the results of flow cytometry (*Fig. 2*). The ICAM-1 expression in CAPAN-2 and PANC-1 were significantly enhanced to 1.5-5 fold of untreated control by the pretreatment with 2.0-50.0 ng/ml of TNF-α in dose dependent fashion (*Fig. 2A*). On the other hand, the high expression of ICAM-1 on CAPAN-2 and PANC-1 were decreased dose-dependently and the expression on cells pretreated with 20.0 ng/ml of TGF-β1 were significantly suppressed to 54.8 and 62.7% of untreated CAPAN-2 and PANC-1 respectively. The expression on SW1990 was also decreased significantly in the fewest range by the pretreatment with 4.0 and 20.0 ng/ml of TGF-β1 (*Fig. 2B*).

Modulation of ICAM-1 mRNA level on Northern Blot Analysis

All three cell lines were stimulated by the treatment with TNF-α in their ICAM-1 mRNA level. After TNF-α treatment, the level of ICAM-1 mRNA was increased than untreated control. A larger transcript, considered as immature ICAM-1 mRNA, was also demonstrated after TNF-α treatment. On the other hand, slightly decreased but no significant change in ICAM-1 mRNA expression was found after TGF-β1 treatment compared with untreated control (*Fig. 3*).

Figure 3. **Northern blot analysis of ICAM-1 mRNA level in pancreatic cancer cells after treatment with TGF-β1 (20 ng/ml) or TNF-α (50.0 ng/ml). Lane1:SW1990 no treatment, 2: SW1990 TGF-β1 treatment, 3: SW1990 TNF-α treatment, 4: CAPAN-2 no treatment, 5: CAPAN-2 TGF-β1, 6: CAPAN-2 TNF-α, 7: PANC-1 no treatment, 8: PANC-1 TGF-β1, 9: PANC-1 TNF-α. ICAM-1 mRNA was detected as 3.3 k.b. transcript (arrow). A larger transcript was detected after TNF-α treatment in each cell lines (arrow head)**

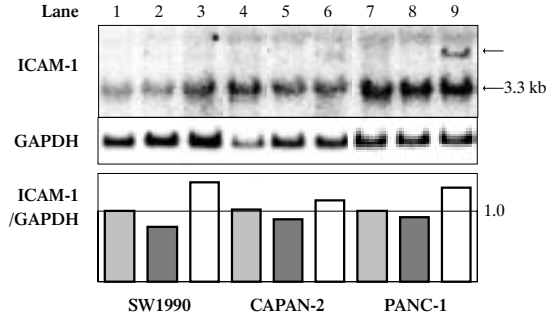
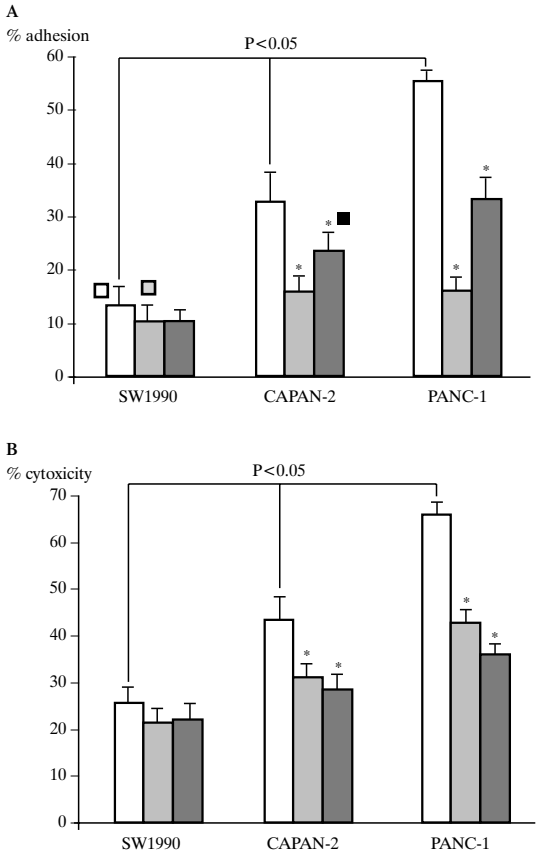


Figure 4. **Effect of anti-ICAM-1 neutralizing antibody and TGF-β1 on adhesion of lymphocytes to cancer cells (A) and cancer cell cytotoxicity by lymphocytes (B). Cancer cells were pretreated without (□) or with anti-ICAM-1 neutralizing antibody (2 μg/ml) (▨) or TGF-β1 (20 ng/ml) (■) for 48 h prior to assay. (*P<0.05)**



Effect of TGF-β1 on adhesion and cytotoxicity by PBMLs

The adhesion of PBMLs to SW1990 and SW1990 cytotoxicity by PBMLs were significantly lower than CAPAN-2 or PANC-1, corresponding to the lowest ICAM-1 expression of SW1990. While the high adhesion of PBMLs to CAPAN-2 or PANC-1 and these cancer cell high cytotoxicity by PBMLs were significantly inhibited by the addition of anti-ICAM-1 neutralizing antibody (2 μg/ml), these high adhesion and cytotoxicity were also significantly inhibited by the treatment with TGF-β1 (20 ng/ml), which is consistent with the results of decreased ICAM-1 expression by TGF-β1 (*Fig. 4*). In SW1990 cells, the adhesion and cytotoxicity were slightly inhibited by anti-ICAM-1 neutralizing antibody and TGF-β1, but significant difference was not observed.

Effect of TGF-β1 on *in vivo* liver metastatic assay

SW1990 which has low expression of ICAM-1 demonstrated high metastatic ability in 9 of 10 mice (90%) under untreated condition, but CAPAN-2 and PANC-1 which show relatively high ICAM-1 expression, demonstrated low incidence of liver metastasis, 18.2% and 0% respectively under untreated condition. After pretreatment with TGF-β1, the metastatic potential of CAPAN-2 was significantly enhanced from 18.2% to 84.6% (11 of 13 mice) and TGF-β1 increased not only the incidence but also the number of metastatic nodules in CAPAN-2. TGF-β1 enhanced the metastasis of SW1990 from 90 to 100%, but PANC-1 cells did not induce any liver metastasis even by the treatment with TGF-β1 (*Fig. 5*).

Discussion

ICAM-1 is expressed on various type of cells, including white blood cells, fibroblasts, endothelial and some epithelial cells. Although the expression of ICAM-1 is found on various cancer cell surface, low ICAM-1 expressing cancer cells demonstrate resistance to lysis by effector cells and correlate to their malignant and metastatic potential compared with highly ICAM-1 expressing cells [7]. Soluble ICAM-1 molecules (sICAM-1) have been known to be released by ICAM-1 expressing cells and elevated serum levels of sICAM-1 have been identified in various inflammation, infection and also malignant diseases. The elevated release of sICAM-1 by cancer cells have been reported to block the recognition of cancer cells by T lymphocytes and let them escape from host immune defense [11]. These immunosuppression may be suggested to induce cancer progression and metastasis.

As we investigated ICAM-1 expressions on three pancreatic cancer cells by flow cytometry and ELISA assay in the present study, SW1990 cell which has lowest ICAM-1 expression revealed highest liver metastatic ability in nude mice splenic injection model [12]. On the other hand, CAPAN-2 and PANC-1 cell which have high ICAM-1 expression demonstrated weak or no liver metastatic potential. These present results suggested that the decreased ICAM-1 expression on pancreatic cancer cells correlated with their metastatic potential, which were consistent with other previous reports [7].

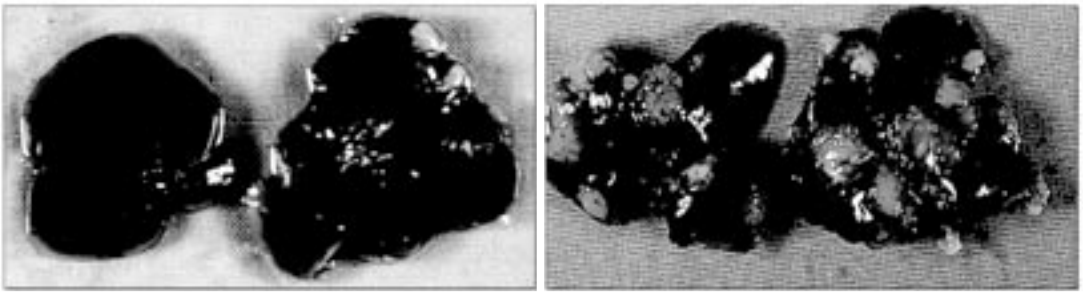
The ICAM-1 expression on cancer cells have been reported to be induced by various cytokines, and TNF-α has also known to be a strong inducer of its expression and involved in the metastatic process at host microenvironment on several steps

***Figure 5*. Effect of TGF-β1 on *in vivo* liver metastasis. Upper table is the ratio of liver metastasis bearing nude mice after splenic injection of pancreatic cancer cells treated with or without TGF-β1. Lower figure is macroscopic findings of the resected liver in nude mice injected of CAPAN-2 cells**

Effect of TGF-β1 on *in vivio* liver metastatic ability;
the ratio of liver metastasis bearing nude mice

	no treatment		TGF-β1 treatment
SW1990	90.0% (9/10)		100% (10/10)
CAPAN-2	18.2% (2/11)	P<0.05	84.6% (11/13)
PANC-1	0% (0/5)		0% (0/5)

5 weeks after splenic injection



Control of CAPAN-2 TGF-β1 treatment of CAPAN-2

[4,7]. Increased ICAM-1 expression on cell surface may lead a convenient circumstance for the cancer cell recognition by host effector cells. While we investigated the modulating effect of multifunctional TGF-β1 in ICAM-1 expression on pancreatic cancer cell, TGF-β1 significantly induced decreasing ICAM-1 expression on all cell lines. According to the immune system, this behavior of TGF-β1 might be a disadvantage in the recognition of cancer cells by cytotoxic effector cells and the supplement of TGF-β1 may possibly increase the metastatic ability.

Our present study also demonstrated that TGF-β1 treatment slightly decreased ICAM-1 mRNA expression level in all cell lines, but its mRNA modulation was not significantly inhibited compared to the decreasing effect in ICAM-1 protein expression. This results suggested that little influence was supposed to be obtained by TGF-β1 on transcriptional regulation of ICAM-1 expression, and modulation in ICAM-1 expression by TGF-β1 might be limited to post-transcriptional level, such as the manner or the location of ICAM-1 protein existence.

While TGF-β1 was found to be increased in tissue remodeling after acute pancreatitis, pancreatic cancer sometimes accompany inflammatory change by obstructing exocrine pancreatic duct which may be involved in increased production of TGF-β1 [13]. TGF-β1 generally acts to inhibit cell proliferation through binding to its receptor on cell surface, and the loss of these growth suppression induced by deficient receptor function has been known to be involved in carcinogenesis [14]. Furthermore, TGF-β1 produced in autocrine by tumor cells and in paracrine by host parenchymal stromal cells at microenvironment were reported to be implicated in host immunosuppression and also involved in tumor progression and metastasis by modulating tumor biological characteristics [5].

In addition to these effect, the present study suggested that TGF-β1 may inhibit the immune defence function against not only host effector cells but also cancer cells by modulating ICAM-1 expression. Besides the decreasing ICAM-1 expression on cancer cells by TGF-β1, the adhesion of PBMLs to cancer cells and consequent cancer cell cytotoxicity by PBMLs were apparently inhibited by TGF-β1 treatment of cancer cells as well as the additional treatment with anti-ICAM-1 neutralizing antibody, and these modulation of immunogenicity in cancer cell induced by TGF-β1 was found to be correlated to the enhancement of *in vivo* liver metastasis in CAPAN-2 cells. This function must be newly nominated in one of the multifunction of TGF-β1 and be implicated in the mechanism of highly metastatic behavior of pancreatic cancer. Highly metastatic SW1990 demonstrated slight increasing liver metastasis by pretreatment with TGF-β1, but no enhancement of metastasis was observed in PANC-1. PANC-1 basically reveals the lack of the requiring factors for the accomplishment of liver meatstasis such as expression of sialyl-Le^a or -Le^x , u-PA high activity [8,12] and others, therefore that is supposed to be the reason for no enhancing metastasis by TGF-β1 in PANC-1, despite of the decreasing ICAM-1 expression.

In conclusion, the present study suggested that decreased ICAM-1 expression induced by TGF-β1 contributes to cancer cell escape from immunological recognition and cytotoxicity by effector cells, and this biological TGF-β1 function may play an important role as one of unknown function in the tumor progression and metastatic mechanism of pancreatic cancer. Since TGF-β1 has been known to reveal other invasion or metastasis enhancing functions besides the current ICAM-1 decreasing effect, the inhibiting therapy of these TGF-β1 functions might be one of the new possible approach to inhibit or treat the metastasis of aggressive pancreatic cancer.

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The role of positron emission tomography (PET) in diagnostics of gastroenteropancreatic neuroendocrine tumours (GEP NET)

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Abstract

PET is a successful modality to detect cancer and in recent years has demonstrated a great diagnostic value in large series of tumour types. PET combines high sensitivity and reasonable resolution, and offers the ability to perform whole body scans. ^{18}F-deoxyglucose (FDG)-PET has also been used to diagnose tumours of neuroendocrine origin. Even if ^{18}F-FDG has been successfully and widely employed in oncology, it has not demonstrated a significant uptake in well differentiated neuroendocrine tissues. Thus ^{18}F-FDG is not a good tracer for neuroendocrine tumours, as FDG-PET imaging of number of GEP tumours revealed increased glucose metabolism only in less differentiated GEP tumours with high proliferative activity and in metastatising MTC associated with rapidly increasing CEA levels. In such a situation, additional ^{18}F-FDG PET should be performed only if somatostatin receptor scintigraphy (alone or with ^{99m}Tc-DMSA) is negative. On the contrary, other positron emitter tracers seem to be more promising. ^{68}Ga-DOTA-NOC (tetraazycyclododecanetetraacetic acid-[1--Nal^3]-octreotide) has been used as a positron emitter tracer for the detection of NETs in preliminary studies. A serotonin precursor 5-hydroxytryptophan (5-HTP) labelled with ^{11}C has shown an increased uptake in carcinoids. This uptake seems to be selective and some clinical evidence has demonstrated that it allows the detection of more lesions with PET than with CT or octreotide scintigraphy. Another radiopharmaceutical in the development for PET is ^{11}C-L-DOPA, which seems to be useful in imaging endocrine pancreatic tumours.

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Neuroendocrine gastroenteropancreatic tumours belong to the so called APUD-cell system (amine precursor uptake and decarboxylation) and are consequently capable of producing and processing amines and bioactive peptides [1,2]. Clinical symptoms can be reduced by the use of somatostatin analogs and in vitro the combination of octreotide and dexamethasone has shown additive effects in reducing serotonin release from midgut tumours cells [3]. Malignant neuroendocrine tumours (NETs) constitute a rare and heterogeneous group of tumours including the neuroendocrine adrenal, as well as endocrine islets within glandular tissue (thyroid or pancreatic) and cells dispersed between exocrine cells, such as endocrine cells of the digestive and respiratory tracts, known as carcinoid tumours [4]. They have traditionally been classified further according to the anatomical site of origin: foregut, midgut and hindgut [5]. Whithin these subgroups the biological and clinical characteristics of them vary considerably [6]. NETs can either be sporadic or occur as part of familiar syndromes, mainly Multiple Endocrine Neoplasia (MEN) I and II, von Hippel Lindau (VHL) syndrome and neurofibromatosis (NF)-I, and have multipotent secretory capacities producing distinct clinical syndromes [7-9].

In Poland the availability of PET-CT diagnostic is low. There are only a few PET study centers, e.g. in Bydgoszcz. Positron emission tomography (PET) imaging is based on the individual characteristics of cancer tissue (proliferative activity, viability, other biological parameters). PET is a successful modality to detect cancer and in recent years, it has demonstrated a great diagnostic value in large series of tumour types [10]. PET combines high sensitivity and reasonable resolution, and offers the ability to perform whole body scans. Positron emission tomography (PET) supplies a range of labeled compounds to be used for the characterization of tumour biochemistry. In PET, to diagnose neuroendocrine tumours, radiopharmaceutics like fluorine-18--fluorodeoxyglucose (^{18}F-FDG), ^{11}C-harmine, nuclear antigen Ki-67, tetraazycyclododecanetetraacetic acid-[1-Nal^3]-octreotide

(^{68}Ga-DOTA-NOC), ^{11}C-5-hydroxytryptophan (^{11}C-5-HTP), ^{11}C-L-DOPA are usually used. The PET-technique provides visualization, but also the assessment of tumour biological characteristics such as substrate transport, metabolism, receptor expression and enzyme activity [3].

The metabolism of tumour cells is characterized predominantly by enhanced glycolysis [11]. The first routinely used PET tracer in oncology, ^{18}F-labeled deoxyglucose (FDG), was successfully used for diagnosis of cancer, reflecting increased expression of glucose transporter in cancerous tissue. Cancer cells demonstrate increased glucose metabolism, due in part to an increased number of glucose transporter proteins and increased intracellular enzyme levels of hexokinase and phosphofructokinase, among others, both of which promote glycolysis [12]. Fast FDG uptake in physiologic glucose utilization reduces serum insuline levels to near basal levels, and thus diminishes FDG uptake in such organs as heart. This tracer, however, usually does not show sufficient uptake in well-differentiated tumours such as neuroendocrine tumours. Fluorine-^{18}F-deoxyglucose (^{18}F-FDG), a glucose analogue, is a marker of tumour viability, based upon the increased glycolysis that is associated with malignancy in comparison with most normal tissue [13]. ^{18}F-FDG is not a good tracer for neuroendocrine tumours, as FDG-PET imaging of a number of GEP tumours revealed that increased glucose metabolism can only be seen in less differented GEP tumours without somatostatin receptors and with high proliferative activity and in metastasing medullary thyroid cancer associated with rapidly increasing CEA levels [14].

^{18}F-deoxyglucose (FDG)-PET has also been used to diagnose tumours of neuroendocrine origin. Therefore, this examination provides opposite information to that from ^{111}In-pentetreotide or $^{123/131}$I-MIBG, which is related to the tissue differentiation. The most clinically aggressive neuroendocrine tumours have shown an intense uptake of FDG. The sensitivity of FDG-PET in these malignancies with a poor prognosis seems to be higher than in SST receptor scintigraphy. To detect some metastatic lesions, that are not revealed by other conventional modality techniques, ^{18}F-FDG diagnostics staged and monitored medullary thyroid carcinomas, phaeochromocytomas and paraganglioma [15-18]. Using [^{111}In-DTPA-D-Phe]-pentetreotide for in vivo somatostatin receptors imaging, no malignant lesions could be visualized. Primary tumours and all metastases showed an increased FDG uptake, suggesting high glucose tumour metabolism. The use of ^{11}C-harmine-PET enables diagnostic visualization of the tumour cells, mainly in subpopulations of neuroendocrine GEP-tumours such as non-functioning endocrine pancreatic tumours (EPT), small tumour lesions and somatostatin receptor negative tumours [19]. The number of specific neuroendocrine markers and hormones expressed by these tumours, or the amount of biologically active neurohormones secreted, is not prognostically indicative of malignant behaviour. Ki-67 is a nuclear antigen expressed in proliferating cells (G1, S, G2 and M phases) but not in resting cells (G0 phase). Well-differentiated gastroenteropancreatic tumours demonstrated inverse relationships between cell proliferation (GEP tumour tissue low profilerative activity – low Ki-67 expression), in vivo in somatostatin receptors expression and FDG uptake (normal biodistribution). High levels of Ki-67 immunoreactivity were observed in all primary tumours and metastases of less well-differentiated GEP tumours (no in vivo somatostatin receptor expression), in a direct proportion to the increased FDG uptake [11].

^{11}In-DTPA-octreotide (Octreoscan) is the diagnostic agent classically used in preliminary phase to assess the biodistribution of the therapeutic compound, based on binding to the SST_2 receptor subtype. For PET studies, ^{68}Ga-DOTA-TOC has been used as a positron emitter tracer. ^{68}Ga-DOTA-NOC (tetraazycyclododecanetetraacetic acid-[1-Nal^3]-octreotide) has been synthesised by Wild and co-workers. This compound for PET imaging has high affinity for SST_2 and SST_5 and has been used for the detection of NETs in preliminary studies. As ^{68}Ga-DOTA-NOC binds to NETs via a receptor mechanism, the sensitivity of this compound could be lower than of ^{18}F-DOPA, which accumulates via a metabolic mechanism, in some histological types expressing a low number of somatostatin receptors. The overall sensitivity and specificity of ^{68}Ga-DOTA-NOC as compared to those of ^{18}F-DOPA have still to be assessed, but it may be realistic to predict a complementary role for the two tracers, as they explore different features of NETs [20].

On the contrary, other positron emitter tracers seem to be more promising. New radiopharmaceuticals base on different precursors, namely 5-hydroxytryptophan (5-HTP) and L-DOPA labelled with ^{11}C [21,22]. A serotonin precursor 5-hydroxytryptophan (5-HTP) labeled with ^{11}C has proved successful with neuroendocrine GEP tumours: increased uptake in carcinoids and demonstrated increased uptake and irreversible trapping of this tracer in carcinoid tumours, the tracer was shown both in primary tumours and in metastases [10,22]. The uptake was so selective and the resolution was so high that one could detect more liver and lymph node metastases with PET than with CT or octreotide scintigraphy. Another radiopharmaceutical in the development for PET is ^{11}C-L-DOPA, which seems to be useful in imaging of endocrine pancreatic tumours [10]. ^{11}C-L-DOPA was applied as a tracer for dopamine synthesis, and it was successfully used to detect both functioning and non-functioning endocrine pancreatic tumours [22]. PET can detect small lesions in the thorax and the abdomen not detected by other methods. It detects more lesions in the liver and lymph nodes than other methods and furthermore, it can be used to monitor treatment effects.

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Tartrate-resistant acid phosphatase 5b and its correlations with other markers of bone metabolism in kidney transplant recipients and dialyzed patients

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Abstract

Purpose: Renal osteodystrophy is a common complication of chronic renal failure and renal replacement therapy. Successful kidney transplantation reverses many of these abnormalities, however, the improvement is often incomplete. The osteoclast specific 5b isoform of tartrate-resistant acid phosphatase (TRAP) 5b has recently been proposed a specific and sensitive marker of bone resorption. The aim of the study was to assess correlations of TRAP 5b with markers of bone resorption and formation in kidney transplant recipients, hemodialyzed and peritoneally dialyzed patients and healthy volunteers.

Material and methods: We assessed PTH, markers of bone formation-alkaline phosphatase and its bone isoform, osteocalcin, markers of bone resorption – procollagen type I carboxy-terminal extension peptide, procollagen type I cross-linked carboxy-terminal telopeptide, serum CrossLaps-Ctx, β2--microglobulin and urinary deoxypyridynoline (DPD), expressed as DPD/creatinine ratio. (BMD) bone mineral density measurements were determined for femoral neck and lumbar spine (L2-L4) using DEXA.

Results: In dialyzed patients markers of bone formation and resorption were significantly higher than in healthy volunteers, whereas in kidney transplant recipients these disturbances were less pronounced. TRAP 5b correlated positively with age and mainly with markers of bone resorption in kidney transplant recipients, dialyzed patients and healthy volunteers. TRAP 5b did not correlate with BMD in any groups studied.

Conclusion: Since TRAP 5b correlated mainly with markers of bone resorption, it may serve as a new additional marker of bone resorption in the assessment of renal osteodystrophy.

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Introduction

Renal osteodystrophy is a common complication of chronic renal failure and renal replacement therapy. Unfortunately, monitoring for the presence and progression of renal osteodystrophy remains problematic. Bone biopsy, a gold standard for diagnosis of renal osteodystrophy, is rarely performed mainly due to patients refusal. Thus, considerable efforts have been devoted to the development of reliable non-invasive methods to assess bone metabolism in uremic patients [1,2]. Successful kidney transplantation reverses many of these abnormalities, however, the improvement is often incomplete. Over the last several years various biochemical markers of bone metabolism have been proposed [3].

Bone metabolic status can be determined by measuring the amount of various biochemical markers of bone resorption and formation in urine and serum samples. The degree of bone formation rate is assessed by measuring plasma levels of bone-specific alkaline phosphatase (bALP), osteocalcin or procollagen type I carboxy-terminal extension peptide (PICP) [3]. On the other hand, osteoclasts resorb bone by secreting acid and lysosomal proteases to the space between cell membrane and bone matrix. Acid dissolves hydroxyapatite and proteases remove and degrade organic components (type I collagen and other matrix proteins) from the matrix. Therefore, pyridinoline (PYD), deoxypyridinoline (DPD), cross-linked N-terminal telopeptides of type I collagen and C-terminal telopeptides of type I collagen (ICTP), degradation products of C-terminal telopeptides of type I collagen (CrossLaps) and tartrate-resistant acid phosphatase (TRAP) have been proposed as markers of bone resorption [3]. Osteoclasts secrete TRAP into the circulation during bone resorption. Human serum contains two forms of TRAP, 5a and 5b, of which 5b is derived from osteoclasts, and 5a from some other, yet unindentified source [4]. Newly developed

immunoassay, specific for TRAP 5b, may be a specific method for the determination of bone resorption rate from serum samples [4,5]. So far there are no data about TRAP 5b in kidney allograft recipients and their correlation with other markers of bone resorption and formation. The majority of studies on renal osteodystrophy dealt with hemodialyzed patients, while a relatively low number have studied kidney allograft recipients and patients on (CAPD) chronic ambulatory peritoneal dialysis; extremely rarely these conditions have been studied comparatively.

Taking all this into consideration the aim of the study was to assess a new marker of bone resorption – TRAP 5b and its correlation with other markers of bone metabolism and BMD in kidney transplant recipients and dialyzed patients.

Materials and methods

47 kidney allograft recipients (age range 26-63 years), with stable graft function (serum creatinine 1.6±0.7 mg/dl), no infection (C-reactive protein within the normal ranges), and liver dysfunction (normal prothrombin time and normal activities of alanine and asparaginine aminotransferases) were enrolled in the study. The immunosuppressive therapy consisted of cyclosporine (the average concentration was 151±52 ng/ml), azathioprine and prednisone. Patients were engrafted for a period of 1 to 10 years (mean time 48±28 months). The average time on dialyses before renal transplantation was 38±19 months.

Two groups of clinically stable dialyzed patients were also included in the study: 45 chronically hemodialyzed patients and 25 peritoneal dialysis patients maintained on CAPD. The causes of renal failure among HD patients varied between chronic glomerulonephritis (n=26), chronic interstitial nephritis (n=12), polycystic kidney disease (n=3) and other or unknown causes (n=4). In CAPD patients renal failure was due to glomerulonephritis (n=13), chronic interstitial nephritis (n=7), polycystic kidney disease (n=2) and other or unknown causes (n=3). All the patients were offered a choice as to the prefered dialysis modality after demonstration of both methods. Dialyzed patients were included in the study on the basis of having residual renal function (necessary to assessed urine DPD).

In HD patients blood was drawn in the morning between 8.00 and 9.00 am. to avoid circadian variations before the onset of dialysis session (and heparin administration) and after hemodialysis from the arterial line of hemodialysis system immediately before discontinuation of the extracorporeal circulation. Ultrafiltrate samples were also taken. All the patients had required regular hemodialyses for 4-5 h a day 3 times a week. Blood flow was usually 150-200 ml/min with a dialysate flow rate of 500 ml/min. Ultrafiltration was varied according to patient's actual weight. Among all HD patients, 41 subjects were dialyzed on polysulphone membranes (Fresenius, Bad Homburg, Germany) and 4 on cuprophane membranes (Gambro, Nipro, Braun or Terumo dialyzers). All the patients were dialyzed with bicarbonate dialysates.

In CAPD subjects blood samples were drawn in the morning when subjects, receiving their normal diet, appeared for routine office assessment of dialysis therapy after an overnight oral fast. The same applied for kidney transplant recipients. All the CAPD patients were performing four 2 l exchanges. They were using Baxter Twin Bag system or Fresenius Andy Plus system with low dialysate calcium concentration (1.25 mmol/l or 1.00 mmol/l, respectively). Dwell times were generally 4-6 h during the day and 8 h overnight. The osmotic pressure of CAPD fluid was adjusted in accordance with the extent of ultrafiltration in each patient. The patients height and weight were recorded for all groups.

At the time of the study, all the dialyzed patients were also receiving concomitant drugs as calcium carbonate (dose ranged from 3 to 11 g/d) and alphacalcidol (0.25 μg/d). None of the patients had received aluminium hydroxide or other drugs known to affect bone metabolism. None of the patients had any history of fractures. All the patients were Caucasians. None of female patients were postmenopausal. None of them were receiving estrogen therapy.

All the patients were informed about the aim of the study and gave their consent. The study was approved by Local Ethical Committee. Healthy volunteers served as a control group to establish the normal ranges for the markers of bone metabolism. Regional BMD were measured by a Lunar DPX bone densitometer according to the principle of DEXA. BMD measurements were determined for femoral neck and lumbar spine (L2-L4).

Intact PTH, osteocalcin concentrations were studied by radioimmunoassay using kits from Cis, France. Levels of PICP (procollagen type I carboxy-terminal extension peptide) and ICTP (procollagen type I cross-linked carboxy-terminal telopeptide) were estimated by radioimmunoassay kits from Orion Diagnostica, Finland. Serum CrossLaps-Ctx were assayed by ELISA using commercially kit from Osteometer, Denmark. Concentration of β2-microglobulin was measured using kits from AlphaDialab, Vienna, Austria. Bone-specifc alkaline phosphatase was estimated using commercially available kit from Metra Biosystem, USA. Creatinine concentration in serum and urine was assayed by means of a standard laboratory method. DPD in urine was assayed by immunochemiluminescence (ACS 180 Bayer) and expressed as DPD/creatinine ratios. Serum TRAP 5b was estimated using commercially available kit from Suomen Bioanalytika Oy, Finland. Data were analyzed using Statistica 6.0. computer software (Pearson or Spearman correlations).

Results

All the clinical and biochemical data are presented in the *Tab. 1*. In kidney transplant recipients concentrations of PTH, osteocalcin, ICTP, serum CrossLaps, PICP, β2-microglobulin were significantly higher than in healthy volunteers, whereas TRAP 5b, DPD, ALP and bALP were similar to those values obtained in the healthy subjects. In both groups of dialyzed patients PTH, osteocalcin, TRAP 5b, PICP, DPD, Ctx and β2-microglobulin were significantly higher, but concentrations of calcidiol and calcitriol were significantly lower than in the healthy volunteers. Dialyzed patients have higher DPD (only HD), ICTP, Ctx, β2-microglobulin and lower calcidiol and calcitriol than kidney transplant recipients. HD patients have higher DPD, ICTP and lower cholesterol than CAPD patients.

Table 1. **Markers of bone metabolism in the hemodialyzed patients (HD), peritoneally dialyzed patients (CAPD), kidney transplant recipients (Tx), and healthy volunteers (CG)**

	HD n=45	CAPD n=25	Tx n=47	CG n=25
age (years)	51±16	47±15	44±13	43 ±14
BMI (kg/m^2)	23.1±4.2	25.1±3.1	24.8±2.97	24.9±3.5
duration of dialyses (months)/time after Tx	38±29	33 ±24	38±19	NA
cholesterol (mg/dl)	176±46°	214±42**	219±41**	168±34
triglycerides (mg/dl)	119±62*	161±70***	221±43***	89±25
total protein (g/l)	6.40±0.60*	6.21±0.68**	6.82±0.68	6.99±0.65
albumin (g/l)	3.90 ±0.51*	3.52±0.59**	4.17±0.46	4.29±0.45
urea before HD (mg/dl) urea in CAPD, Tx,	119.3±27.2***	120.4±28.5***	99.7±32.2***	19.6±6.2
total Ca (mmol/l)	2.14±0.30##	2.09±0.30##	2.29±0.36	ND
P (mg/dl)	5.59±1.88##	4.83±1.86	4.54±1.59	ND
vit D_3 (ng/ml)	11.3±8.0***###	9.9±6.9***###	56.0±17.1	55.9±14.7
$1,25(OH)_2D_3$ (ng/ml)	12.8±5.1***###	12.7±5.8***###	27.9±8.8***	64.0±11.8
PTH (pg/ml)	319±300***	287±202***	129±102***	33±11
Osteocalcin (ng/ml)	171±102***##	172±90***##	99±61**	23±5
ALP (U/l)	152±116	101±68	100±51	ND
bALP (ng/ml)	45.0±31.5	49.0±31.0	31.6±21.2	18.1±5.5
PICP (μg/L)	238.9±114.8*	265.8±132.1*	213.4±105.4*	147.9±42.1
TRAP (U/L)	1.79±1.12*	1.94±1.63*	1.54±1.40	1.08±0.80
DPD (nM/mmol)	11.9±8.0***##°	7.1±4.1**	5.5±4.0	4.9±1.8
ICTP (μg/L)	42.6±18.0***#°	37.1±17.0***#	11.8±8.0***	2.6±08
Ctx (pmol/L)	14737±8694**#	15297±9984**#	10535±6945*	3079±1293
β2-microglobulin (μg/mL)	14.9±6.2***#	12.5±3.8***#	6.5±3.8*	2.2±0.7
BMD – lumbar spine – score (kg/m^2)	1.01±0.18	1.15±0.20*#	1.03± 0.17	ND
BMD – femur neck T-score (kg/m^2)	0.84±0.26	0.85±0.38	0.89± 0.14	ND

Values given are means ± SD; * p<0.05, ** p<0.01, *** p<0.001 vs control group; # p<0.05, ## p<0.01 ### p<0.001 vs Tx; ° p<0.05; HD vs CAPD; ND – not done, NA – not applicable

In our study in kidney transplant recipients TRAP 5b correlated significantly both with a marker of bone formation – ALP (r=0.34, p=0.01) and resorption (ICTP r=0.41, p=0.01, β2-microglobulin r=0.36, p=0.01) as well as with PTH (r=0.42, p=0.005). Moreover, TRAP 5b correlated with current concentration of cyclosporine (r=-0.30, p=0.04), current dosage of prednisone (r=-0.41, p=0.009), azathioprine (r=-0.33, p=0.01). In HD patients TRAP 5b correlated significantly with age (r=-0.33, p=0.028), time on dialyses (r=0.55, p=0.0001), serum albumin (r=0.30, p=0.046), Kt/V (r=0.45, p=0.002), PTH (r=0.40, p=0.008). Correlations between TRAP and total protein (r=0.27, p=0.077), phosphorus (r=0.27, p=0.077), alkaline phosphatase (r=0.29, p=0.054), bone-specific alkaline phosphatase (r=0.29, p=0.06), pH (r=-0.28, p=0.071) almost reach statistical significance. In CAPD patients TRAP 5b correlated with age (r=-0.43, p=0.029), albumin (r=0.38, p=0.047), ICTP (r=0.44, p=0.33). In the healthy volunteers TRAP 5b correlated with age (r=0.55, p=0.02), ICTP (r=0.47, p=0.045), β2-microglobulin (r=0.63, p=0.012). TRAP 5b did not correlate with BMD in any studied groups.

Discussion

So far, data about bone metabolism and its assessment in kidney transplant recipients are limited, particularly in comparison with dialyzed patients, both HD and CAPD. In the recent study, Durieux et al. [6] reported that serum $25(OH)D_3$, PTH and urinary N-telopeptides excretion were normal in kidney transplant recipients. Cruz et al. [7] reported that in kidney allograft recipients either high-bone turnover or normal bone turnover was observed. Patients with high-bone turnover exhibited lower BMD at the lumbar spine, and the hip and higher PYD or DPD and/or osteocalcin. In our study we assessed also a new marker of bone resorption – TRAP 5b. The osteoclast specific 5b isoform of TRAP has recently been proposed as a sensitive and specific marker of bone resorption. Recently, a new immunoassay was developed to estimate only TRAP 5b. Halleen et al. [4] have shown that serum TRAP 5b measure using this assay is a much more specific and sensitive marker of bone resorption than total serum TRAP [4]. In a recent paper, Halleen et al. [5] reported that changes in serum TRAP 5b after 6 months of hormone replacement therapy, determined by this immunoassay, correlated significantly with the changes of all markers of bone turnover determined, including serum N- and C-terminal propeptides of type I collagen and urinary-free deoxypyridinoline. They also reported an elevated TRAP 5b in patients with osteoporosis [5]. Moreover, in their study, performed on early postmenopausal women TRAP 5b showed a significant negative correlation with BMD. In our study in neither kidney transplant recipients nor dialyzed patients (both HD and CAPD, evaluated together or separately) TRAP 5b did not correlate with BMD.

Up to date, there are no data about TRAP 5b in kidney transplant recipients and its correlations with other markers

of bone metabolism. In patients on peritoneal dialyses TRAP 5b highly correlated with bALP, PTH and osteocalcin in the preliminary report of Poege et al. [8]. Nowak et al. [9] also described a statistically significant correlation between TRAP 5b and PTH in dialysed patients (both hemodialyzed and peritoneally dialyzed). In our study TRAP 5b correlated with PTH only in kidney transplant recipients and HD patients. In our study TRAP 5b correlated significantly with markers of resorption as well as with PTH. Moreover, TRAP 5b correlated negatively with current concentration of cyclosporine, current dosage of prednisone, azathioprine in kidney transplant recipients. According to Minisola et al. [10] serum CrossLaps (betaCtx) seems to be characterized by a superior sensitivity relative to TRAP 5b measurement, at least in the disorders studied (chronic renal failure, established osteoporosis, primary hyperparathyroidism, glucocorticoid excess etc.). However, we have reported previously that serum CrossLaps correlated with urine DPD and serum ICTP in CAPD subjects [11], such correlations were not observed in hemodialyzed patients. We have also observed that serum CrossLaps correlated positively with PTH, markers of bone formation – bALP, osteocalcin, PICP, and positively with ICTP – a marker of bone resorption [12]. Due to this dual correlations – with markers of bone formation and resorption, value of Ctx in assessment of renal osteodystrophy remains to be established.

In the study of Poege et al. [8] TRAP 5b did not differ between hemodialyzed and peritoneally dialysed patients. In our study, TRAP 5b in CAPD and HD patients was significantly higher when compared to the healthy volunteers. TRAP 5b in kidney transplant recipients was within normal ranges.

A reported by Minisola et al. [10] and Nakasato et al. [13] TRAP 5b activity in patients with chronic renal failure and maintenance hemodialyses was not significantly increased, but TRAP 5b concentration in HD patients were significantly higher than in the healthy volunteers. Nakasato et al. [13] suggested the presence of subpopulations of HD patients with increased TRAP 5b acitivity. As reported previously [8,9]. TRAP 5b was not affected by age, gender and time on dialysis in kidney transplant recipients, whereas in dialyzed patients (both HD and CAPD) and in the healthy volunteers TRAP 5b correlated positively with age as previously reported Minisola et al. [10] but only for healthy women. In the study of Lopez Gavilanes et al. [14], on patients with chronic renal failure (without hepatopathy) and 9 hemodialysis patients (with hepatopathy), good relation between PICP, total TRAP (not TRAP 5b) and the biochemical indexes of bone activity and PTH suggested the clinical values of these markers in the follow-up of renal osteodystrophy. There were any differences in levels of markers of bone metabolism between groups with and without liver damage, in spite of the fact that PICP and TRAP were cleared mainly by the liver. In our study patients with liver dysfunction were excluded from the study. Previously, only total TRAP was assayed as in the study of Lopez Gavilanes et al. [14]. Since it is a thermolabile enzyme, the results of its determination were sometimes difficult to interpret.

Concluding, since TRAP 5b correlated mainly with markers of bone resorption, it may serve as a new additional marker of bone resorption in the assessment of renal osteodystrophy.

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The effect of extracorporeal efferent detoxication (EED) methods inclusion in the severe community-acquired pneumonia (CAP) treatment

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Abstract

Purpose: To assess the clinical efficacy including of EED methods in the treatment of severe CAP with endogenic intoxication syndrome.

Material and methods: Severe CAP in patients (n=103, aged 18-60 years, male 89%) were randomly subdivided into the 4 comparable groups. The 1st group (n=30) was standard treated with antibiotics. The 2nd group (n=27) underwent additionally 3 courses of extracorporeal ultraviolet light-exposure (UVLE). The 3rd group (n=25) was co-treated with 3 courses of biospecific hemosorption (BSS). The 4th group (n=21) underwent 2 additional courses of BSS plus 3 courses of UVLE. The effectiveness of these schemes therapy was assessed by clinical and laboratory data.

Results: The additional application of EED methods led to faster disappearance of clinical symptoms, focal chest signs and the infiltrate resolution in chest X-ray (CXR) as compared with standard treatment. Mean time of the disappearance of fever and sweating was 2.2; 2.5; 2.0 days and 4.8; 5.0; 4.6 days in the 2nd, 3rd and 4th group respectively after EED courses vs 6.1 and 8.0 days in 1st group ($P<0.05$). The baseline elastase activity was elevated by 5 times in the 1st-4th groups vs the control (healthy) group and was decreased by 1.5; 2.1; 4.1; 6.6 times respectively ($P<0.05$) after treatment in these groups. The initial trypsin-like activity was increased about 5.5 times in all groups vs control and decreased after therapy by 2.1; 3.8; 6.4; 6.0 times ($P<0.05$) in the 1st-4th groups respectively. Small CXR residual changes persisted in 7, 4 and 5 patients from the 2nd, 3rd and 4th groups vs 12 patients in the 1st group. The spirometry data were normalized faster in the patients who underwent EED methods (by the 14th day) vs the 1st group.

Conclusions: Additional using of EED methods in severe CAP therapy is more effective (as compared with traditional management with antibiotics only) in term of faster improvement of patients general condition, reduced time of inflammatory infiltrate resolution and hospitalization by 3-4 days. It has been shown that EED methods correct the main pathogenic mechanisms of severe CAP. Our results indicated on the EED methods as an attractive supportive therapy for the empiric antibiotics treatment of this disease.

Key words: community-acquired pneumonia, extracorporeal efferent detoxication, elastase, trypsin-like activity, oxidative stress.

Introduction

CAP is a common acute illness. Its incidence in different population has ranged from 2 to 15 cases per 1 000 persons per year [1]. CAP is potentially fatal if not managed appropriately. Thus, CAP mortality rate has ranged from 5% to 15% among hospitalized patients [2]. This figure rises to >50% for patients with severe CAP that requires treatment in intensive care unit (ICU) [3].

Insufficient effect of antibacterial therapy of this disease with severe endogenic intoxication syndrome demands the search of new and more effective methods of treatment. Efforts to improve the efficacy of treating patients with CAP have been focused predominantly on improved schemes of empiric antibiotics therapy. EED methods (EUVE or BSS) have been successfully used in the therapy of acute pancreatitis [4,5] and other severe pathologic conditions [6,7]. However, their clinical efficacy as a supportive therapy to antibiotic treatment of severe CAP has not been studied as of yet.

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For the successful management of severe CAP patients are necessary not only drugs, having the anti-inflammatory effect, but also the abilities to the normalization of microcirculation and oxidation-reduction balance and to the improvement of ventilation as well. One of such a method is the autotransfusion of UVLE blood. The bactericidal and oxygenic effects of UVLE have been known since a long time [8]. Recently the interest in this method has been increased. It was promoted by the high observable costs of long antibiotics therapy as well as suppression of immunity, dysbacterioses and toxic liver lesion.

In an effort to provide clinicians with the better management of severe CAP, we would like to increase the efficacy of this pathology treatment. Thus, we conducted the prospective randomized controlled trial to investigate the hypothesis that the additional treatment of severe CAP with EED methods would result in a better outcome as compared with the conventional scheme as well as to ensure that there is no an increase in the adverse events from such an intervention. The present studies aimed at determining whether treating of severe CAP patients with the addition of EED methods is superior to the standard therapy with antibiotics alone.

Material and methods

Patients

We examined 103 inpatients (11 female, 92 male) with severe CAP (aged from 18 to 60 years, who were admitted and treated in ICU of the 1st, 2nd and 10th Minsk large public hospitals. These patients fulfilled the inclusion criteria and were randomized to this trial between 2000-2003 years. Dominated men (89%), among them there were 70% smokers. 7% of these patients were taken β-lactams prior to the hospital admission during 1-2 day. 90% of the patients were admitted to the hospital within 1-3 days of severe CAP onset. This diagnosis was defined by the new focal signs and symptoms of the lower respiratory tract infection (complaints of cough, expectoration, dyspnoea, chest pain) as well as by clinical history, physical examination of chest on admission. The results of initial biochemical analyses (hematocrit, leucocytes count, renal function, sodium and potassium values and arterial blood gases), laboratory (including respiratory tract microbiology) examination, CXR findings (new pulmonary infiltrate that were not attributable to other causes) and associated pathologies were considered.

Severe CAP was considered if one or more of the following criteria were present: hemodynamic instability – systolic BP<90 mmHg or diastolic BP<60 mmHg, heart rate (HR) >100 beats/min; new onset of mental impairment; increased respiratory effort (respiratory rate – RR>30/min); multilobar involvement; presence of significant pleural effusion; acute renal failure; leucopenia (<4000/μl) or severe leucocytosis (>30000/μl); anemia; hypoalbuminemia or bacteriemia.

The inclusion criteria for the study were established before the trial and strictly followed. These inclusion criteria were: >18 and ≤60 years old, clinical and laboratory signs of severe CAP, confirmed new lung infiltration by CXR (compared with old radiographs if available) and disease onset outside of hospital. Exclusion criteria were: pulmonary infiltrates due to other forms of pneumonia (nosocomial or due to large-volume aspiration and against the background immune deficiency status); age of the patients >60 years; presence of an infiltrate on CXR typical for the pleural effusion; presence of severe coexisting diseases – cancer (in previous 5 years), diabetes mellitus, lung tuberculosis and chronic obstructive pulmonary disease as well as chronic diseases of heart (coronary artery disease or congestive heart failure of the III-IV grade according to NYHA), liver (preexisting chronic hepatitis, cirrhosis), pancreas (chronic pancreatitis), kidneys (preexisting chronic renal failure with documented abnormal serum creatinine level >180 μmol/L outside of pneumonia episode); immunosupression (receiving chemotherapy or treatment by immunosuppressive drugs).

This study was approved by the Human Studies Committee of the Belarusian State Medical University and informed consent has been obtained from these patients.

Procedures

The ultraviolet light exposure (UVLE) of blood was performed with the apparatus "Nadezda" in the one-time polymeric cannula in the oscillating regimen. The patient's blood was exposed to ultraviolet light twice: at taking blood and then during returning blood to the body. The volume of UV exposed blood was 2-2.5 ml/kg of body mass at one procedure. The rate of blood taking made up 18 ml per min. The normal saline solution (NaCl 0.9%) was a blood stabilizer with addition of heparin in a dose of 50-70 units per kg of the body mass. The course of blood photomodification in addition to the standard treatment included 3 procedures of autotransfusion of UV-radiated blood in the 2nd and 4th groups. We performed one ultraviolet photomodification of blood daily with an interval of one day.

The biospecific hemosorption (BSS) with antiprotease hemosorbent "Ovosorb" (containing ovomucoid, which could bind the serum proteinases) [9,10] by using of a peristaltic pump was performed during the first days on admission. This procedure was used after the preliminary heparinization of the body by intravenous administration of heparine on the basis of 150±25 units per kg of the patient's body mass. The vein-venous type of connection was used. The rate of perfusion was 50-60 ml per min and the time of perfusion – from 60 to 90 min. The course of treatment consisted of 3 manipulations (with additional using of BSS) in the 3rd group and 2 procedures in the 4th group. BSS was done by single sorption daily with an interval of a day.

The therapy effectiveness was assessed by clinical, commonly used laboratory tests and CXR data, including severity assessment – by the clinical severity index (CSI) [11] and by the scale PORT [12].

The study design

All the patients were initially divided into 4 comparable groups according to: sex (χ^2=0.01; P>0.05); age (χ^2=6.88; P>0.05); class (χ^2=0.58; P>0.05) and clinical severity index (χ^2=1.16; P>0.05 among these groups); baseline CXR as well as host immune status, concomitant chronic diseases and alcohol consumption. There were no significant differences in antimicrobial treatment regimens (including time of receiving the first antibiotic delivery and door-to-drug delivery time) among the patients of these groups. The physicians in charge received no

Table 1. **Distribution of patients from the 1st-4th groups according to age**

Age (years)	Groups			
	1st n=30	2nd n=27	3rd n=25	4th n=21
<20	1	3	1	1
21-30	8	6	6	5
31-40	3	6	6	4
41-50	11	7	4	6
51-60	7	5	8	5
Mean age	40.5±2.4	37.5±2.3	40.5±2.6	39.9±2.8

The difference in mean age among these groups was not significant (P>0.05)

Table 2. **Distribution of patients in the 1st-4th groups according PORT (Fine score)**

Severity class (scores)	Groups			
	1st n=30	2nd n=27	3rd n=25	4th n=21
IV (91-130)	25	23	21	19
V (>130)	5	4	4	2
Mean score	108.5±3.8	106.7±3.8	103.1±3.6	104.9±3.8

The difference in the mean scores among these groups was not significant (P>0.05)

information on the objectives or specific target variables of this trial, although they could not be completely blinded. We did quasi randomized trial to compare four schemes of therapy. Thus, on admission the patients were randomly assigned to one of the four groups according to the therapy schemes depending on No of case record.

The 1st group comprised of 30 patients (27 men and 3 women), receiving the standard empirical treatment within the first hours of admission, which included antibiotics. This treatment began with intravenous β-lactamase-stable or third-generation cephalosporin alone or their combination with intravenous macrolide. Mostly patients received their first dose within 8 h (if no clinical effect was detected, intravenous "antipneumococcal" fluoroquinolone were taken), acetic acid derivates, mucolytics as well as (under the indications) physio- and oxygen therapy were given. The 2nd group was formed by 27 patients (24 men and 3 women), who underwent additionally UVLE courses. The 3rd group included 25 men (22 men and 3 women) treated in addition with procedures of BSS. Twenty one (19 men and 2 women) patients (the 4th group) were additionally treated with courses of UVLE plus BSS. No deaths occurred among the patients of these groups during treatment.

The control group for the biochemical parameters was formed by primary blood donors (16 healthy men and 5 women; with mean age 38.6±2.5 years).

Methods

Bedside spirometry was measured in the upright position at a fixed time (9:00) using a computered portable spirometer "Spirovit SP-10" ("Shiller"). The best FEV_1 and FVC after three reproducible measurements were used in the analysis.

We used the two levels principle of the immune status estimation. We regarded to the tests of 1st level the following tests: calculation of leucocytic formulas, detection of T-lymphocytes, immunoglobulins (Ig) and nonspecific resistance of the body (phagocytosis). We specified the lesion localization of the link of subpopulations T-lymphocytes by the second level tests – T-helpers (CD^{+4}) and T-supressors (CD^{+8}).

The quantitative and functional states of the immune system were determined in dynamics: on admission, by the 12--14th day and by the 21st day. The number of T-B lymphocytes with method of spontaneous rosset-formation T-helpers and T-supressors by the method of immunefluorescention with monoclonal antibody receptors were done [13]. The levels of serum immunoglobulins (Ig) were performed by Mancini's method of radial immunodiffusion [14]. Complement level (by 50% haemolysis of sensitized erythrocytes), circulating immune complexes, leukocyte phagocytary activity and leucocytic intoxication index were estimated [13,14].

The measurements of elastase [15] and trypsin-like activity, α_2-macroglobuline (α_2-MG) as well as α_1-antitripsine (α_1-AT) level by the complex method [16] and level of the peptide substances belonging to a group of "middle molecules" (MM) [17] were carried out.

The activity of catalase [18] and superoxide dysmutase (SOD) [19] as well as the level of malondialdehyde (MDA) [20] were assessed in the erythrocytes, as the indicators of the "oxidative stress".

Statistical analysis

The data are shown as mean ± SEM unless otherwise indicated. The paired and unpaired t-test was used to test the significance of baseline characteristics of the 1st-4th groups as well as the treatment effects within the groups and between them. All P values were two tailed. The group comparison used the Student's t test for quantitative variables and χ^2 method Fisher's exact test for qualitative variables. The χ^2 or Fisher exact test was used to compare categoric variables. The non-parametric Mann-Whitney U test was used to compare the difference between unpaired samples. The results of study were further corrected using the Bonferroni method where necessary. The significance level was set at $P \leq 0.05$.

Results

The 1st group did not differ according to the age and gender from the 2nd-4th groups additionally treated with supportive EED methods. Severe CAP was more often (29%) observed in the patients ranging from 41-50 years (*Tab. 1*). Periodic alcohol abuse (>80 g of calculated absolute ethanol per day at least during last year) without signs of dependence in history was detected in 1/3 of these patients. Two third of the patients have had a cold preceding this CAP onset. The work was connected to stay outside in 1/5 of these patients.

All the patients were stratified for the prediction of risk

Table 3. **Baseline selected clinical features of patients admitted to ICU**

Clinic signs	Groups				χ^2
	1st n=30	2nd n=27	3rd n=25	4th n=21	
Body temperature:					
38.1-39°C	7	8	6	5	1.96; P>0.05
>39°C	20	16	18	15	
Respiratory rate:					
21-29 breaths per min	23	22	19	14	1.44; P>0.05
≥30	7	5	6	7	
Heart rate:					
<109 beats per min	15	12	16	11	7.21; P>0.05
110-124	10	8	7	6	
BP systolic <90 mm Hg	9	8	4	5	6.99; P>0.05
Weakness	19	18	18	17	2.02; P>0.05
Productive cough	25	19	18	13	3.03; P>0.05
Haemoptysis	8	6	4	5	0.93; P>0.05
Pleural pain	17	13	16	16	4.19; P>0.05
Dullness (at percussion)	16	19	20	13	4.72; P>0.05
Respiration (at auscultation):					
bronchial	7	6	7	8	
hard	5	8	4	4	3.77; P>0.05
weaked	18	13	14	9	
Crackles,	22	20	19	18	1.26; P>0.05
crepitation,	11	10	12	9	0.94; P>0.05
pleural murmur	10	12	11	10	1.30; P>0.05
Pleural effusion	5	4	4	2	0.58; P>0.05

classes and evaluation of the pneumonia severity according to the scale PORT (*Tab. 2*) and CSI (it made up 4.0, 4.2, 4.1 and 4.2 conventional units in 1st-4th groups respectively). The statistical analysis did not detect any difference between the patients of these groups according to these tests.

The acute clinical symptoms and signs of these patients on admission are presented in *Tab. 3*. So, this disease development was sharp in 2/3 of patients in contrast to its gradual development in 1/3 of patients. Constant high fever and single chill were detected before the admittance to hospital in most patients. We observed the severe endogenic intoxication syndrome in most patients, resulting in the development of additional symptoms and vital signs abnormalities (that were associated with severe CAP): high fever (92%), chills (75%), weakness, hypotension (25%), disorders of mental status (17%), hypo- or adynamia (28%), septic shock (6%), vomiting (6%), convulsive syndrome (4%), syncope (8%), negative reaction from another body's systems (toxic hepatitis, nephropathy, myocardial dystrophy and collapse). One third of the patients had various arrhythmias. All the patients had an abnormal CXR (unilobar or multilobar alveolar infiltrate). The baseline radiological features were similar in all groups. Chest radiographic patterns were the following: bilateral transient non-malignant infiltrate was marked in 1/3 of patients (2 lobes in 25% of them and more than 2 lobes in 8%), unilateral infiltrate was noted in 67% of patients. Severe CAP was located in the right and left lung in 57% and 43% of the patients respectively. Pleural effusion was detected in 15% of these patients (*Tab. 3*).

The normal count of leucocytes was detected in the peripheral blood only in 10% patients on admission and leucopenia was observed in 5% of them. The mean leucocytes count made up $12.3\pm0.9x10^9$/L; 12.2 ± 1.2; 12.1 ± 1.3 and 13.1 ± 1.4 in the 1st-4th groups respectively (P>0.05 among these groups). Leucocytosis was marked in 85% of the patients (in intervals of: 10.1–15.0x10^9/L in 59% and >15.0x10^9/L in 21% of patients). Shift to the left of neutrophils was detected in 85% of the patients and toxic neutrophils granularity – in 36% of them. The leucocytosis and shift to the left were accompanied by significant increase of ESR as well as leucocytic index of intoxication. The latter was achieving 6.4 ± 1.8; 7.2 ± 2.0; 6.0 ± 0.7 and 4.0 ± 0.5 conventional units in 1st-4th groups respectively (P>0.05 among these groups). The mean ESR value made up: 42 ± 3; 39 ± 2; 45 ± 3 and 40 ± 3 mm/h in 1st-4th groups respectively (P>0.05 among these groups). Thus, increase of ESR >20 mm/h was observed in 95% of the patients.

Severe CAP on admission was verified by the changes of the biochemical tests too. Thus, increase of fibrinogen level (>4.5 g/L) and C-reactive protein (CRP) were detected in 93% and 89% of patients respectively. Transitory significant increase of AST and ALT was revealed in 35% of the patients. Some of the patients had toxic nephropathy in the first week of hospitalization. Thus, increased blood level of urea (due to intensification of catabolic processes) and transitory proteinuria were detected in 26% and 38% of patients respectively.

In all patients, the sputum and blood microbial investigations were done: the first sputum Gram stain (as a guide to the initial therapy), the sputum culture and the estimation of its sensitivity to antibiotics. The responsible pathogen was isolated only in 21% of these patients. Thus, *S. pneumoniae* (59%), mixed infections (14%; with *S. pneumonia* as the most commonly involved agent), *S. aureus* (14%), *Klebsiella pneumoniae* (9%) and *Pseudomonas aeruginosa* (4%) prevailed among detected causative pathogens.

Table 4. **Immune parameters of the patients on admission**

Parameter	Patient's groups				
	Control n=21	1st n=30	2nd n=27	3rd n=25	4th n=21
Lymphocytes, %	31± 1	16±2*	15±2*	15 ±2*	15± 2*
Lymphocytes×10^9/L	1.7±0..2	1.7±0.2	1.6±0.1	1.5± 0.1	1.9± 0.2
T-lymphocytes, %	60.7±0.9	61.0±2.2	64.0±2.4	63.3±2.6	61.0±2.9
T-lymphocytes×10^9/L	1.14±0.02	1.08±0.11	1.16±0.11	0.98±0.10	1.11±0.12
active T-lymphocytes, %	25.5±0.6	29.8±2.2	26.1±2.3	26.4±2.3	27.6±2.4
active T-lymphocytes × 10^9/L	0.45±0.01	0.50±0.05	0.46±0.05	0.41±0.05	0.50±0.06
B-lymphocytes, %	6.1±0.3	6.1±0.6	7.1±0.6	6.0±0.6	7.1±0.7
B-lymphocytes ×10^9/L	0.17±0.02	0.11±0.02*	0.13±0.01	0.09±0.01*	0.13±0.02
CD^{+4}	54.7±1.2	46.3±2.0*	44.9±2.5*	40.1±3.5*	46.6±2.2*
CD^{+8}	13.9±0.9	18.4±1.2*	19.5±1.6*	18.2±2.0*	17.8±1.5*
Ig G, g/L	10.9±0.7	15.7±1.5*	14.6±1.0	17.8±1.7*	14.3±1.9
Ig A, g/L	3.2±0.2	4.6±0.4*	4.3±0.4*	4.4±0.4*	4.8±0.6*
Ig M, g/L	0.7±0.1	1.9±0.2*	1.6±0.2*	1.9±0.2*	1.6±0.2*
CH_{50}, hemolytic U.	53.0±0.7	59.3±3.6	56.5±3.8	62.3±2.7	56.8±4.9
Immune complexes, conv.U	7.7±1.3	8.1±1.2	7.1±0.9	8.4±1.0	7.6±1.5
Leucocytes phagocytic activity, %	58.9±2.2	62.4±3.2	54.1±3.3	56.4±3.5	57.4±2.3
Phagocytosis (latex-test), conv. U	1.16±0.05	1.10±0.02	1.10±0.02	1.11±0.02	1.11±0.02

* – P<0.05 vs the control; conv. U – conventional units

The baseline changes of immune status concerned both cellular (mostly) and humoral links (*Tab. 4*). These data were verified by the expressed and various disorders of immune response of severe CAP patients during first days of disease onset both on mean values and number of the patients with deviation from the control. Thus, relative lymphopenia, deficiency of absolute count of B-lymphocytes were detected before treatment in these patients. Though the level of peripheral blood T-lymphocytes in these patients did not differ from the control (as compared with the mean value), the initial decrease (<1.0x10^9/L) of the count of these cells was marked in: 57%, 37%, 57% and 38% of patients the 1st-4th groups respectively (χ^2=1.32; P>0.05). The count of T-lymphocytes exceeded 1.2x10^9/L in 33%, 48%, 28% and 52% of patients from the 1st-4th groups respectively (χ^2=1.79; P>0.05 among these groups).

Although, the baseline relative count of active T-lymphocytes in these groups did not significantly differ from the control one, the deficiency of active T-lymphocytes (<0.4x10^9/L) was observed in 40%, 48%, 52% and 40% of patients from the 1st-4th groups respectively (χ^2=0.50; P>0.05). Meanwhile, the increase of active T-lymphocytes count (>0.5x10^9/L) was marked in 40%, 37%, 28%, 48% patients of the 1st-4th groups respectively (χ^2=0.89; P>0.05 among these groups). Deficiency of T-helper (CD^{4+}) cells and an increase in the mean number of T-suppressors (CD^{8+}) were observed in these patient's groups in contrast to the control. Thus, we detected the low baseline of CD^{+4} level as well as the high its level in 20, 19, 16, 14 and in 7, 6, 3, 6 patients of 1st-4th groups respectively. The high initial count of CD^{8+} cells was detected in 19, 17, 13, 10 patients of 1st-4th groups respectively.

The changes of humoral response have been shown by an increase in the serum levels of IgG, M, A in all patients groups as compared with the control one. Thus, the high initial level of IgA as well as the low its level were observed in 20, 14, 15, 13 and 3, 12, 6, 5 patients from 1st-4th groups respectively. The high baseline levels of IgG and IgM were marked in 17, 16, 17, 7 and 21, 15, 22, 15 patients of 1st-4th groups respectively.

The complement titer was low (<50 hemolytic units) in 13%, 8%, 8%, 14% of patients of 1st-4th groups respectively (χ^2=0.81; P>0.05 among these groups). Meanwhile, an increase of the complement titer (>55 hemolytic units) was marked in about 70% patients of all groups. The leucocyte phagocytic activity in patients from all groups did not differ from the control one. Thus, its decrease (<1.09 conventional units) was observed (*Tab. 4*) in 53%, 38%, 52% and 38% of 1st-4th patients groups respectively (χ^2=0.72; P>0.05 among these groups).

The balance of the lipids peroxidation (LP) and an antioxidant defense in the blood was characterized by a significantly increased baseline level of MDA – the main toxic product of LP membranes (by about 30%) as well as activity of SOD (by about 1.7 times) in all groups as compared with the control one (P<0.05). On the contrary, initial catalase activity was decreased (by about 10%) significantly in all groups vs the control one (*Tab. 5*).

A predominant part of the patients on admission had some imbalance in the system of proteinase-inhibitors. Thus, data showing a significant increase in serum elastase activity (by about 5 times in all groups vs the control one) and trypsin-like activity (by about 5.5 times in all groups vs the control one) against a background of α_1-AT and α_2-MG deficiency are listed in *Tab. 6*. So, an increase of elastase activity (>12.6 μmol/h/L) was detected in 93%, 100%, 92 % and 95% of the 1st-4th patient's groups respectively (χ^2=0.05; P>0.05 among these groups).

The enhancement of trypsin-like activity was revealed in 87%, 74%, 88% and 95% of the 1st-4th patient's groups respectively (χ^2=0.37; P>0.05 among these groups). α_1-AT

Table 5. **Condition of system of lipids peroxidation/antioxidant defense in patient's groups on admission**

Parameter		Patient's groups				
		Control n=21	1st n=30	2nd n=27	3rd n=25	4th n=21
SOD	U/ml of blood	508±27	841±35*	841±45*	799±39*	843±54*
	U/mg of Hb	4.1±0.2	7.6±0.4*	7,3±0.4*	7.3±0.5*	7.9±0.8*
Catalase	μmol H_2O_2/μL x min	10.4±0.3	9.1±0.4*	9.3±0,3*	9.3±0.3*	8.9±0.4*
MDA	μmol/ml of hemolysate	3.5±0.2	4.3 ±0.2*	4.3±0.2*	4.3±0.2*	4.4±0.2*
	μmol/mg of Hb	1.01±0.05	1.29±0.06*	1.29±0.04*	1.37±0.09*	1.36±0.09*

* – P < 0.05 vs the control; U – units; μL – microlitr; μmol – micromol

Tab. 6. **Condition of system proteases-inhibitors in patient's groups on admission**

Parameters	Patient's groups				
	Control n=21	1st n=30	2nd n=27	3rd n=25	4th n=21
Elastase, mmol/h x L	9.8±1.3	55.7±4.7*	49.8±3.0*	49.8±6.3*	49.1±4.6*
Trypsin-like activity, nmol/sec x L	28.7±3.6	154.7±33.1*	157.0±30.9*	159.8±34.0*	158.3±39.4*
α_1-AT, μmol/sec x L	7.7±0.4	3.2±0.7*	3.3±0.7*	2.8±0.7*	2.8±0.7*
α_2-MG, μmol/ sec x L	0.92±0.04	0.41±0.06*	0.51±0.07*	0.46±0.06*	0.43±0.08*
Middle molecules, g/L	0.38±0.03	0.96±0.05*	1.08±0.05*	1.12±0.06*	1.07±0.07
Total protein, g/L	70.7±1.3	64.2±1.6*	60.8±1.2*	62.1±0.7*	62.8±1.6*

* – P < 0.05 vs the control; μmol – micromol; nmol – nonamol

level before treatment was decreased by 2.4; 2.2; 2.8; 2.9 times (p<0.05 vs the control group) in the 1st-4th groups respectively. Thus, the deficiency of α_1-AT was observed in 83%, 85%, 88% and 90% of the patients from of 1st-4th groups respectively (χ^2=0.05; P>0.05 among these groups). The mean baseline level of α_2-MG was reduced by about 2 times in all groups vs control one (P<0.05). A decrease of α_2-MG level was marked in 83%, 74%, 80% and 81% of the 1st-4th patient's groups respectively (χ^2=0.09, P>0.05). We detected also a significant increase in the level of MM and the lower concentration of total protein as compared with the control group. Thus, a decrease of albumin level (<35 g/L) was observed in 27%, 33%, 20% and 24% of the patients from 1st-4th groups respectively (χ^2=0.74; P>0.05 among these groups).

The assessment of pulmonary function was available only in 78 patients and it showed mainly a restrictive ventilation disorders of various degree severity. This testing was impossible in remaining 22 patients due to expressed chest pain or intoxication psychosis. Analysis of pulmonary function detected abnormal lung function indices. Thus, we revealed decrease of FVC (<85% from norm) in: 84%, 83%, 91% and 87% of patients from the 1st-4th groups respectively (χ^2=0.06; P>0.05). 36%, 39%, 48 % and 67% of the patients from the 1st-4th groups respectively had reduced FEV_1 (<80% from norm; χ^2=3.30; P>0.05 among these groups) (*Tab. 7*).

We also estimated the dynamics of basic clinical features before-post carrying out EED methods as well as just after ending the first procedure. All used EED methods (better BSS plus UVLE) reduced the endogenic intoxication signs (decrease of HR, RR, BT and CSI) just after using of one procedure. Treatment with EED methods was associated with shorter time of fever resolution as compared with the conventional therapy.

Table 7. **Bedside spirometry of patient's groups on admission**

Parameters	Patient's groups			
	1st n=25	2nd n=24	3rd n=23	4th n=15
VC, %	70±3	74±3	71±3	69±5
FVC, %	64±3	65±4	64±3	65±4
FEV_1,%	74±4	71±4	71±4	70±5
FEV_1/FVC	88±4	90±3	92±3	88±4
FEV_{25},%	71±7	68±6	76±7	59±5
FEV_{50},%	74±5	68±5	72±5	65±6
FEV_{75},%	77±6	67±6	71±5	67±7

Differences among these groups were not significant (P>0.05)

Thus, high fever (BT>38°C) and low grade fever were observed in 61% and 39% of patients from 4th group before carrying out BSS plus UVLE. Significant decrease of BT (accompanied with the improvement of general health status) was observed in 58% of these patients (from those in 3 patients BT was normalized) just after finishing carrying out a single UVLE plus BSS procedure. BT did not change just after using BSS only in 42% of patients. Normalization of BT was observed in dynamics (in 10 hours) in 86% of patients of the 4th group and a day later in remaining 14%. Thus, BT made up 36.8+0.1°C (P<0.05 vs the initial data) following day. Dynamics of BT decreasing did not differ significantly after single using of UVLE, BSS and UVLE plus BSS (*Tab. 8*).

Tachypnoea (>25 breaths per min) was decreased by 22% in patients (vs the baseline data) after carrying out single BSS plus UVLE procedure. Thus, 43% of these patients noted significant relief of breathlessness in the early postsorption period. Initial

Table 8. **Dynamics (before-after the procedure) of basic clinical parameters after single carrying out of UVLE, BSS and BSS plus UVLE**

Parameter	UVLE		BSS		BSS plus UVLE	
	Before/after		Before/after		Before/after	
BT°C	38.3±0.6	37.8±0.5*	38.0±0.1	37.5±0.1*	38.4±0.2	37.2±0.1*
RR, breaths/min	26.4±0.6	25.7±0.3*	25.7±0.7	23.7±0.6*	26.1±0.5	20.3±0.3*
HR, beats/min	110±2	103±2*	112±1	107±2*	114±2	102±2*
Systolic BP, mmHg	111±2	113±2	112±3	114±2	109±3	112±2
CSI, conv.U	4.2±0.2	3.7±0.1*	4.1±0.2	3.8±0.2*	4.2±0.1	3.5±0.2*

* – P<0.05 vs the initial level

breathlessness at rest and during conversation was marked in 5 patients of the 4th group. After the BSS plus UVLE procedure it disappeared and these patients could start to move independently within the nearest 10 hours. The rest 57% of patients have felt a reduction of breathlessness and a relief of breath within a day.

Single application of BSS plus UVLE caused a more significant decrease of RR, than the separate using of these procedures. Thus, RR in a day after using single combination of BSS plus UVLE also decreased by 10% and made up 18 breaths per min (P<0.05 vs the initial level and the similar values in the 2nd-3rd groups).

The positive influence of BSS plus UVLE procedure has concerned HR too (it was decreased by 11% after this manipulation). Thus, HR in patients of 4th group in a day decreased by 5% (made up 97 beats per min; P<0.05 vs the initial value, but P>0.05 vs the similar parameter of the 2nd and 3rd groups). While, HR decreased by 6% and 4% during single application of UVLE or BSS.

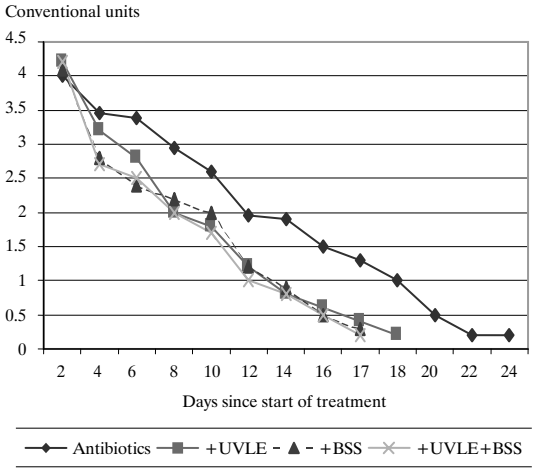
Systolic BP did not significantly change in all groups just after finishing a single EEDM procedure. Systolic BP every other day exceeded (by 11%) the initial level in the 4th group and made up 122 mmHg (P<0.05 vs the initial data; but P>0.05 vs similar value in the 2nd and 3rd groups).

86% of patients noted reduction of weakness after a single application of BSS plus UVLE. Productive cough appeared instead of dry cough in 38% of these patients as well as disappearance of cough was detected in 14%. Out of 16 patients who initially had a pleural pain, its reduction was noted in 4.

CSI just after a single application of BSS plus UVLE was decreased by 17%. CSI every other day was reduced by 3% and made up 3.4±0.2 conventional units (P<0.05 vs the initial value; but P>0.05 vs the similar value of the 2nd and 3rd groups). CSI was decreased (*Fig. 1*) by 2.5 times by the 10th day of treatment (P<0.05) in patients who were treated with the application of BSS+UVLE.

Further decrease of CSI (by 2 times) was marked by the 14th day of therapy. CSI made up 0.23±0.01 unit in the 4th group by the 17th day. Comparison of CSI values has not revealed the significant difference in different terms of treatment of patients who were applied the other EED methods. Meanwhile, decreasing of CSI was slower after traditional therapy. Thus, CSI was decreased by 1.5 times by the 10th day (made up 2.6±0.1 units; p<0.05 vs the value in the 4th group) and CSI has achieved value 0.53±0.04 unit only by the 22 day of conventional management.

Figure 1. **Dynamics of CSI during standard treatment as well as the additional courses of UVLE, BSS and UVLE plus BSS**



The analysis of immune response in the period of using different schemes of severe CAP therapy showed that the similar dynamics of leukocytes count was observed in various terms of treatment in all groups. Nevertheless, leukocytosis (leukocytes count >9.0x10^9/L) persisted in 6 patients from the 1st group by the 21st day of therapy, in 2 from the 2nd group and in 1 patient from the 3rd group. Meanwhile, such leukocytosis was not observed in patients from the 4th group (χ^2=9.08; P<0.05).

Baseline relative lymphopenia was observed in patients of all groups on admission. A significant increase of lymphocytes level was noted in all groups just by the 12-14th day as compared with the initial level. The relative mean number of rosset-formated and active T-lymphocytes as well as rosset-formated B-lympocytes and complement titer in patients of all groups did not significantly change during treatment. But, qualitative analysis showed, that the normalization of low baseline level of T-lymphocytes (%) was detected in 6, 5, 7, 9 patients of the 1st-4th groups respectively. From the patients with the high baseline level of B-lymphocytes (%) as well as the low level, its normalization was detected in 6, 6, 6, 8 and 9, 6, 7, 7 patients of the 1st-4th groups respectively.

The additional using of EED methods eliminated an imbalance of T-helpers/T-supressors ratio by increasing of T-helpers level and by decreasing of T-supressors level just by the 12-14th day. Thus, T-helpers level increased (by 16% and 10%; P>0.05

vs the initial level) by the 22th day of therapy in the 2nd and 4th groups, while it was increased more in the 3rd group (by 28%; P<0.05 vs the initial data). Among the patients with initially low levels of T-helpers, their normalization was noted in 4, 12, 7, 6 patients of 1st-4th groups.

Deficiency of T-helpers persisted during the whole period of standard antibiotic treatment (pre-post – 46.3±2.0 and 43.8±1.6). The baseline mean level of T-supressors was replaced by it's normalization in all groups by the 12-14th day of therapy. Thus, high initial level of T-supressors was replaced by it normalization in 14, 13, 9, 7 patients of 1st-4th groups respectively.

The level of IgG remained increased (by 37%, 32%, 39%, 42%; P<0.05 vs the control) in patients of the 1st-4th groups respectively by the 12-14th day of therapy. On the whole, IgG content had the tendency to normalization to the end of treatment in all groups. Thus, its high baseline level was normalized in 11, 11, 15, 5 patients of 1st-4th groups.

The mean level of IgA was normalized after the therapy in all patients' groups too. Thus, the high baseline level of IgA was replaced by it normalization in 14, 10, 12, 11 patients of 1st-4th groups.

The mean IgM level decreased to some extent, but persisted increased (P<0.05 vs control) after standard therapy in contrast to the significant reduction of it after the additional using of EED methods (by 18%; 31% and 24% in the 2nd-4th groups respectively). Thus, the high baseline level of IgM was normalized in 11, 11, 15, 5 patients of 1st-4th groups.

The level of immune complexes was decreased by 2 times in patients of the 2nd group (P<0.05 vs the initial level) by the end of therapy. Similar (but non-significant) dynamics of immune complexes level was observed in the 3rd-4th group in contrast to the absence of such positive changes during traditional management.

A significant increase of LPA (digested phagocytes activity) was already noted by the 12-14th day of treatment in patients of the 2nd-4th groups (by 26%, 33% and 23% respectively). Whereas, LPA did not significantly change during traditional therapy. Phagocytosis (according to the latex test) did not change in patients of all groups after the therapy.

Using of EED methods caused more significant positive changes of the proteinase inhibitors system (*Tab. 9*). Thus, the high baseline elastase level as well as trypsin-like activity were significantly decreased by 1.5; 2.1; 4.1; 6.6 times and by 2.1; 3.8; 6.4; 6.0 times (P<0.05) after finishing treatment in the 1-4th groups respectively. α_1-AT level was significantly increased by 1.8; 2.2; 3.0; 2.7 times in these groups after the therapy. The level of α_2-MG increased up to normal too in all groups by the end of management.

The baseline high elastase level in patients of the 4th group was decreased by 44% (vs the initial level) by the 12-14th day and still decreased by 72% by the 20-22th day. Similar dynamics of elastase level was marked in patients of the 3rd group too. An additional using of UVLE alone was less effective concerning the decrease of elastasemia (P<0.05 vs the data of the 3rd and 4th groups by the 20-22th day). *Tab. 9* shows, that the high level of elastasemia persisted by the 22th day in the patients who underwent standard therapy (P<0.05 vs other groups).

Using of UVLE alone did not influence the proteinase/ /antiproteinase system. Whereas, a single application of BSS or BSS plus UVLE caused significant decrease of the elastase and trypsin-like activity as well as the MM level. Thus, the elastase and trypsin-like protease activity was decreased by 58% and 61% (P<0.05 vs the initial level) just after the finishing of BSS plus UVLE procedure. We registered some an enhancement of proteinase activity in a day, but it did not reach an initial level. The elastase and trypsin-like activity in a day were increased by 28% and by 22% (P<0.05 vs the initial level) in patients of the 4th group.

The baseline high level of the trypsin-like activity in patients of the 4th group was decreased by 61% by the 12-14th day (p<0.05 vs the initial level) and by 56% by the 22th day (P<0.05 vs the data by the 12-14th day). The trypsin-like activity was normalized in patients from the 3rd and 4th groups by the 20--22th day; meanwhile its high level persisted in the patients who were conventional treated (mostly) or with the application of UVLE.

Using the combination of BSS plus UVLE promoted the increase of the proteinase inhibitors level by the 12-14th day. So, the maintenance of α_1-AT and α_2-MG was increased by 2 times (P<0.05 vs the initial level) and the levels of these proteases inhibitors were completely normalized by the 20-22th day. The contents of α_1-AT and α_2-MG did not significantly change just after the combined BSS plus UVLE procedure every other day too. Similar dynamics of this antiproteinase activity was observed in the 3rd group. While a slight increase of α_1-AT by the 12-14th day was observed in patients of the 1st and the 2nd groups with further enhancement of this level by 45% to the 20-22th day (P<0.05 vs the data by the 12-14th day) in the 2nd group and by 38% in the 1st group (P<0.05 vs the data by the 12-14th day). A significant increase of α_2-MG level was marked only by the 20-22th day in the patients underwent standard management.

The level of MM was decreased by 13% (P<0.05 vs the initial level) after using a single BSS plus UVLE procedure. Meanwhile, MM contents every other day did not change. The MM level was decreased by 2 times by the 12-14th day (P<0.05 vs the initial level) in the 4th group and later – by 1.6 times by the 20-22th day (P<0.05 vs the data by the 12-14th day). Similar changes of the MM content were detected in patients after a single using of BSS or UVLE. Thus, the level of MM was decreased by 2.5 times by the 22-th day after the application of UVLE in contrast to 1.9 times after standard therapy. MM level also significantly decreased by the 20-22th day during antibiotic management, however, it did not reach the control one.

The total protein level had the tendency to an increase in patients who were treated with the application of EED methods (mostly in the 2nd group). On the contrary, the total protein was further decreased in those patients receiving traditional therapy.

The high baseline SOD activity in all groups was significantly decreased in the course of treatment by 1.4 times and 1.6 times in the 1st and the 2nd-4th groups respectively (*Tab. 10*). The decrease of the SOD activity by 30%, 39%, 36% and 39% (P<0.05 vs the data by the 12-14th day) was detected by the 20--22th day in the 1st-4th patient's groups respectively. This reducing of SOD activity was more detected for this period according to recalculation of this value per mg of Hb (by 23%, 30%, 42% and 44% in the 1st-4th patient's groups respectively) (*Tab. 10*).

Table 9. **The condition of proteinase/proteinase inhibitors system in the course of standard antibiotics therapy and with the additional treatment using of EED methods**

Parameter	Control n=21	Standard therapy (ST) (days)			ST+UVLE (days)			ST+BSS (days)			ST+BSS+UVLE (days)		
		Before n=30	12-14th n=30	20-22th n=25	Before n=27	12-14th n=27	20-22th n=24	Before n=25	12-14th n=25	20-22th n=24	Before n=21	12-14th n=21	20-22th n=20
Elastase mmol/h x L	9.8 ±1.3	55.7 ±4.7*	59.2 ±6.1*	38.2 ±3.7*,▽	49.8 ±3.0*	41.7 ±3.8*,⊗,**	23.3 ±2.4*,⊗,**,▽	49.8 ±6.3*	28.4 ±2.3*,⊗,**	12.1 ±1.5⊗,**,▽	48.9 ±4.6*	27.4 ±4.1*,⊗,**	7.6 ±1.2⊗,**,▽
Trypsin-like activity nmol/s x L	28.7 ±3.6	154.7 ±33.1*	120.4 ±18.0*	72.5 ±16.3*	157.0 ±30.9*	107.3 ±26.0*	41.4 ±5.9**,▽	159.8. ±34,0*	60.0 ±10.0⊗,**	24.9 ±3.6⊗,**	158,3 ±39,4*	62.5 ±1.4.2*,**	27.2 ±3.5⊗,**
α_1-AT µmol/s x L	7.7 ±0.4	3.2 ±0.7*	3.6 ±0.8*	5.8 ±0.7**,▽	3.3 ±0. 7*	4.0 ±0.8*	7.3 ±0.7**,▽	2.8 ±0.7*	5.3 ±0.6**	8.3 ±0.6⊗,**,▽	2.77 ±0.7*	5.6 ±0.8**	7.4 ±0.7**
α_2-MG µmol/s x L	0.92 ±0.04	0.41 ±0.06*	0.57 ±0.05*	0.80 ±0.05**,▽	0.51 ±0.07*	0.90 ±0.06⊗,**	1.00 ±0.04**,⊗	0.46 ±0,06*	0.86 ±0.06⊗,**	0.90 ±0.04**	0.43 ±0.08*	0.87 ±0.07⊗,**	1.01 ±0.04⊗,**
Middle molecules g/L	0.38 ±0.03	0.92 ±0.05*	0.73 ±0.05*,**	0,51 ±0.05*,**,▽	1.08 ±0.05*	0.61 ±0.05*,**	0.43 ±0.03**,▽	1.12 ±0.06*	0.54 ±0.04*,⊗,**	0.36 ±0.03⊗,**,▽	1.07 ±0.07*	0.50 ±0.06⊗,**	0.31 ±0.02⊗,**
Total protein g/L	70.7 ±1.3	64.2 ±1.6*	62.1 ±1.0*	62.8 ±1.1*	60.8 ±1.2*	63.8 ±1.3*,**	65.8 ±1.3*,**	62.1 ±0.7*	63.5 ±0.7*	64.8 ±0.8*	62.8 ±0.6*	62.3 ±0.3*	63.5 ±1.5*

* – P < 0.05 vs control group; ⊗ –P < 0.05 vs standard treatment in the same period; ** – P < 0.05 vs data of this group on admission; ▽ –P < 0.05 vs data by the 12-14th day in this group

Table 10. **Dynamics of the parameters of lipids peroxidation and antioxidant defense in the course of standard antibiotic therapy and with the additional treatment using of EED methods**

Parameter		Control n=21	Standard therapy (ST) (days)			ST+UVLE (days)			ST+BSS (days)			ST+BSS+UVLE (days)		
			Before n=30	12-14th n=30	20-22th n=25	Before n=27	12-14th n=27	20-22th n=24	Before n=25	12-14th n=25	20-22th n=24	Before n=21	12-14th n=21	20-22th n=20
SOD	U/ml blood	508 ±27	841 ±35*	802 ±38*	585 ±23**,▽	840 ±45*	910 ±44*,**	513 ±22**,▽	780 ±39*	914 ±20*,**,⊗	501 ±28**,▽	843 ±54*	940 ±51*,⊗	512 ±28**,▽
	U/mg Hb	4.1 ±0.2	7.6 ±0.4*	8.0 ±0.3*	6.6 ±0.2*,▽	7.2 ±0.3*	8.2 ±0.3*,⊗,**	4.8 ±0.3*,⊗,**,▽	7.2 ±0.6*	7.3 ±0.3*	4.2 ±0.3**,⊗▽	7.9 ±0.8*	7.9 ±0.5*	4.4 ±0.4⊗,**,▽
Catalase	µmol H_2O_2 µL x min	10.4 ±0.3	9.1 ±0.4*	9.3 ±0.4	7.6 ±0.3*,**,▽	9.2 ±0.3*	10.6 ±0.3⊗,**	8.0 ±0.4*,▽	9.4 ±0.4*	9.8 ±0.3	10.0 ±0.3⊗♦	8.9 ±0.4*	9.8 ±0.4	10.2 ±0.4⊗,♦
MDA	µmol ml hemolysate	3.5 ±0.2	4.3 ±0.2*	3.8 ±0.2	3.3 ±0.2**,▽	4.3 ±0.2*	4.6 ±0.2*,⊗	3,1 ±0.2**,▽	4.3 ±0.2*	4.3 ±0.2*	3.2 ±0.5**,▽	4.4 ±0.2*	4.3 ±0.2*	3.0 ±0.1**,▽
	µmol mg Hb	1.01 ±0.05	1.29 ±0.06*	1.33 ±0.05*	1.32 ±0.06*	1.29 ±0.04*	1.26 ±0.04*	1.07 ±0.09⊗,▽	1,37 ±0.09*	1.18 ±0.04	0.92 ±0.06**,⊗,▽	1.36 ±0.06*	1.26 ±0.07*	0.90 ±0.05⊗,**,▽

* – P < 0.05 vs control group; ⊗ – P < 0.05 vs the standard antibiotic treatment in the same period; ** – P < 0.05 vs the data of this group on admission; ▽ – P < 0.05 vs the data by the 12-14th day in this group;
♦ – P < 0.05 vs the 2nd group

Table 11. **Duration of basic clinical signs in patients who were standard treated and who underwent the courses of UVLE, BSS and BSS plus UVLE**

Signs	Mean duration (days)			
	Standard therapy (ST)	ST+UVLE	ST+BSS	ST+BSS+UVLE
Fever	**6.1±0.4** n=30	**2.2±0.3*** n=27	**2.5±0.3*** n=25	**2.0±0.3*** n=21
Tachycardia	**11.4±1.0** n=23	**6.7±0.5*** n=23	**6.8±0.5*** n=21	**5.7±0.5*** n=19
Sweating	**8.0±0.6** n=30	**4.8±0.4*** n=27	**5.1±0.5*** n=25	**4.6±0.4*** n=21
Weakness	**8.2±0.6** n=30	**5.6±0.4*** n=27	**5.9±0.4*** n=25	**5.1±0.4*** n=21
Cough	**13.3±0.7** n=30	**10.2±0.8*** n=27	**10.1±0.8*** n=25	**8.6±0.8*** n=21
Pleural pain	**8.3±0.4** n=17	**7.5±0.6** n=13	**8.1±0.6** n=16	**7.2±0.5** n=16
Breathlessness	**4.3±0.3** n=30	**2.4±0.2*** n=27	**2.8±0.2*** n=25	**2.2±0.2*** n=21
Dulness (at percussion)	**13.1±1.0** n=16	**9.6±0.9*** n=19	**10.0±0.9*** n=20	**8.8±0.8*** n=13
Normalization of vesicular breath	**17.1±0.8**** n=30	**14.4±0.6*,**** n=27	**12.8±0.7*,**** n=25	**10.2±0.6*** n=21
Disappearance of accessory respiratory murmur	**15.6±0.8** n=30	**12.9±0.6*** n=27	**13.0±0.8*** n=25	**11.4±0.5*** n=21
Unstable hemodynamics	**4.3±0.4** n=12	**2.7±0.3** n=12	**2.6±0.7** n=5	**2.2±0.4*** n=5

* – P<0.05 vs 1st group; ** – P<0.05 vs 4th group

The catalase activity had even decreased in the 1st group (by 18%; P<0.05 vs the initial level) after therapy, but did not change in the 2nd group and increased by 6% and 13% (P<0.05) in patients of the 3-4th groups (rising to the control level).

The baseline high MDA level was decreased by 17%; 33%; 30% (P<0.05) after using EED methods in the 2nd-4th groups, but did not differ in the 1st group. Thus, the normalization of MDA was detected by the 20-22th day of treatment by EED methods. While, the high MDA level (by recalculation of this value per mg of Hb) persisted in patients underwent traditional therapy.

As seen in *Tab. 11*, the application of EED methods promoted earlier disappearance of some signs of endogenic intoxication (fever, tachycardia, sweating, weakness) and focal chest signs (dullness, accessory respiratory murmus) in contrast to conventional management.

As a whole, using of EED methods caused earlier normalization of BT in comparison with treatment by antibiotics only (P<0.05). Thus, patients of the 2nd-4th groups became afebrile approximately on the 2.0; 2.2 and 2.5 days respectively as compared with 6 days in 1st group.

The application of BSS plus UVLE caused a decrease of HR (<90 beats per min) by the 5.7±0.5 day. Tachycardia persisted twice longer (P<0.05) in patients who were standard treated. A single using of BSS or UVLE also caused earlier normalization of HR, as compared with conventional therapy (P<0.05).

Weakness was observed within 5 days after the combined using of BSS plus UVLE. Meanwhile, patients who were standard treated marked weakness lasted (within 8.0±0.6 days; p<0.05 vs the data of the 4th group). Single introduction of BSS or UVLE into the therapy also promoted earlier disappearance of weakness in contrast to the standard treatment (p<0.05).

Sweating within 8.0±0.6 days was noted in patients of the 1st group. Sweating was marked for a shorter time (within 4.6+0.4 days) in patients from 4th group (P<0.05 vs the 1st one). Cessation of sweating was also noted earlier in the patients who were treated with the application BSS or UVLE (P<0.05), than in patients undergoing standard management.

On admission pleural pain was detected in 16 patients who were treated with the application of BSS plus UVLE. Out of this group, the significant reduction or disappearance of painful syndrome was marked in 44% of patients just after a single carrying out of these procedures. Pain arose again within 10 hours in a third of the patients, but it was not such intensive as before. After these manipulations, half of patients did not note changes in the pain syndrome. Pleural pain persisted 7.2±0.5 days after the combined using of BSS plus UVLE. *Tab. 11* shows, that pleural pain disappeared within the same period in patients of other groups (P>0.05).

Disappearance of cough and breathlessness after the including of EED methods in severe CAP treatment was noted to occur earlier than in patients of the 1st group (these patients complained of cough within 13.3±0.7 days). Thus, using of BSS plus UVLE caused earlier disappearance of cough (by the 8.6±0.8 day). Cough persisted a bit longer in patients with a single application of BSS or UVLE (10.1±0.8 and 10.2±0.8 day respectively; P<0.05). Breathlessness of a variable degree was observed in all patients on admission. Breathlessness persisted within 4.3+0.3 day during conventional therapy. The application of BSS plus UVLE caused earlier disappearance of breathlessness (by the 2.2±0.2 day; P<0.05 vs the 1st group). Similar dynamics of breathlessness was marked during a separate using of UVLE or BSS.

Dullness and accessory respiratory murmur disappeared quicker in patients who were treated with the application of BSS plus UVLE in contrast to who underwent standard management. Thus, dullness at lung percussion persisted 8.8±0.8 days during the BSS plus UVLE using. This dullness was detected till the 9.6±0.9 day of UVLE courses and a bit longer (till the 10.6±0.9 day) during including of BSS (both P<0.05 vs the conventional therapy).

Normalization of vesicular breath in patients of the 4th group was observed significantly quicker (by the 10.5±0.6 day) as compared with patients of the 1st-3rd investigated groups (by the 17.1; 14.4 and 12.8 days respectively). Disappearance of accessory respiratory murmur was observed by the 11.4±0.5 day in patients of the 4th group and longer (by the 15.6±0.8 day; P<0.05) in patients of the 1st group.

***Table 12*. Pre-post dynamics of spirometry tests during standard treatment as well as additional using of UVLE, BSS and BSS plus UVLE**

Parameter	Standard therapy (ST) (n=30)			ST+UVLE (n=27)			ST+BSS (n=25)			ST+BSS+UVLE (n=21)		
	Before	12-14th day	20-22th day	Before	12-14th day	20-22th day	Before	12-14th day	20-22th day	Before	12-14th day	20-22th day
VC, %	70±3	75±3	82±6**	73±3	89±3*	94±2*,**	70±3	84±2*,**	92±1**	66±5	88±3*,**	93±3**
FEV_1, %	74±4	78±3	81±3	71±4	86±3**	90±2**	71±4	81±3**	85±3**	70±5	83±3**	92±2**

* – P<0.05 vs the 1st group; ** – P<0.05 pre-post treatment

The stabilization of systolic BP was observed within 2.2±0.4 day in patients of the 4th group. Meanwhile, it normalization occurred later (by the 4.3±0.4 day) during traditional treatment. A single using of UVLE or BSS promoted shortening of the period of instable hemodynamics, however, significant differences vs the 1st group was not revealed.

Positive clinical dynamics was revealed after a combined using of BSS plus UVLE in 20 patients. Thus, we observed significant improvement of general health condition in the one patient and disappearance of breathlessness, but weakness, sweating and subfebrile BT (in the evenings) persisted. In dynamics (during the ensuing month), we revealed lung bacterial destruction in this patient.

Intermediate (by the 5th day) clinical benefits were detected in 21, 26, 24, 20 patients of 1st-4th groups respectively. The lack of positive clinical effect of standard therapy was detected in 9 patients (absence of positive changes – in 4 of patients and negative clinical dynamics – in 5) in contrast to only one patient in the 2nd-4th groups. We revealed the significant difference according these data among the 1st group on the one hand and the 2nd-4th groups on the other hand. Meanwhile significant difference has not been found between the patient's groups whose were treated by EED methods in all cases of the comparison.

Failure of CAP standard treatment was connected with development of lung destruction (abscess formation) and sacculated pleurisy. Thus, severe CAP was complicated by lung abscess in 5 patients of the 1st group in contrast to the 1 patient from the 2nd-3rd-4th groups. Meanwhile, the statistic analysis did not reveal the advantages of EED methods in contrast to the conventional management by prevention of development of lung abscess in these patients (χ^2=3.65; P>0.05 among these groups).

The destructive lung changes and infiltrative changes persisted both by the 21st day in 1 patient of the 4th group. The positive CXR dynamics took place in the rest patients of this group. Mean terms of infiltration disappears made up 17.7±0.7 days. Small residual CXR changes (mainly, intensification and deformation of pulmonary picture and pleural-diaphragmaticus adhesions) remained in 7, 4, 5 patients of the 2nd-4th groups as compared with 12 patients of the 1st group.

CXR signs of lung infiltration at control research disappeared only in 1 patient after conventional management. Thus, appearance of new infiltrates was detected in 3 patients, destructive changes – in 2 as well as lung abscess – in 3 patients. Reduction of infiltration (according to area and intensity) was noted in all other cases (in 70% patients of the 1st group). Lung infiltration did not detect by the 21st day in 68% of these patients. Radiological resolution of severe CAP begun by the 30th day of therapy (and later) in 5% of patients. Thus, the resolution came later (by the 22.2±0.7 day) after traditional treatment in contrast to the application of BSS plus UVLE (P<0.05).

Full CXR resolution and formation of residual changes were revealed in 13, 17, 20, 15 patients and in 2, 7, 4, 5 patients of 1st-4th groups respectively. Thus, radiological lung residual changes were marked in about half of 1st group patients during discharge from hospital and consist in the following: intensification and deformation of pulmonary picture (7 patients), thickening of the interlobular pleura (2 patients), formation of postpneumonic pneumosclerosis (2 patients) and development of pleural-diaphragmatical adhesions (2 patients). The qualitative analysis of the treatment effects on the residual CXR changes did not revealed significant advantages of any methods of severe CAP treatment (χ^2=6.1; P>0.05 among these groups). In our opinion, the initial radiographic involvement patterns have significant effects on the future resolution of severe CAP.

Efficacy of the treatment was estimated too by the terms of CXR resolution of severe CAP. For this purpose we built the cumulative curves and counted value of the logrange criterion (U_L). Considering the fact, that at the analysis resorted to the plural comparisons for calculation the significance, we accounted the Bonferroni correction as well as the Jets correction for compensation the influence of discrete. The analysis showed the higher efficacy of therapy with EED methods combination in contrast to traditional treatment. So, significant difference was revealed among the 1st group and 3rd group ($U_{L=}$10.62; z=3.76; P<0.05), between 1st and 2nd group (U_L =-8.46; z=2.78; P<0.05) as well as among the 1st and 4th group (U_L=-9.61; z=3.70; P<0.05). Any significant difference between the 2nd-4th groups has not been revealed.

The analysis of spirometry revealed the abnormal lung function indices (mainly restrictive disorders) in severe CAP patients on admission. The dynamics analysis of ventilation has shown its normalization in patients additionally treated with the application of EED methods already by the 12-14th day (*Tab. 12*). Thus, earlier normalization of spirometry parameters was revealed in the 4th group (P<0.05 vs the 1st group). Earlier normalization of spirometry tests was noted only during the additional using of UVLE or BSS. VC more increased (by 21%; P<0.05) after the application of UVLE or BSS. Thus, the FEV_1 of the 2nd group increased from 71±4% on day 1 to 90±2% by the 22th day

($P<0.05$). The same data were received in the 3rd-4th groups (none of the changes in VC, FEV_1 differed significantly among the 2nd-4th groups). Meanwhile significant decrease of VC persisted during using of standard therapy by the 22th day (even before discharge from hospital). VC rose from 70±3% on day one to 82±6% by the 22th day ($P<0.05$) in the 1st group.

Discussion

The central point of severe CAP pathogenesis is a microbial aggression against the background of immunodeficiency, hypoxia, systemic oxidative stress with degranulation of cytoplasmic membranes, going out of proteinases into the blood and the development of endogenic intoxication syndrome [21]. It is a generalized reaction of the body to the microbial pathogen with the involvement of the central nervous system, disorders of hemodynamics and all kinds of metabolism. The disturbances in the focal lung lesion, arising in severe CAP, lead to the blockade of antibiotic access into the pulmonary tissue, disorders of reparative processes, sharp increase of proteinases level and depression of local protective factors [22].

The beginning of an acute inflammatory injury of the lungs is initiated by a complex series of events with the development of disturbances of several systems. The outcome of the inflammatory response (progression or containment) depends within certain limits upon the balance between pro- and antiinflammatory mediators. Thus, we detected that the imbalance in the proteinase/antiproteinase system was defined by a decrease of the level of several natural inhibitors (α_1-AT and α_2-MG) against the background of increase of elastase and trypsin-like activity. This imbalance in the severe CAP was accompanied by an increase of MM level and a decrease of total protein concentration in blood.

The influence of the infectious-toxic factor and hypoxia as well as the presence of an inflammation in this disease set up the conditions for an increase of free radical oxidation of the lipids from cellular membranes. The intensification of PL is an important part of severe CAP pathogenesis [23-25] as well as the high MDA level is a natural process. Disorders in the system of LP/antioxidant defense were characterized by the increase of the contents of secondary of lipid peroxidation products (MDA) and decrease of catalase activity. Thus, the listed above various and significant disorders of different parts of homeostasis were not significantly eliminated by conventional treatment with antibiotics and required an additional correction by EED methods.

We revealed that severe CAP developed the background of various disorders of immune response, among which the relative lymphopenia, decrease of T-lymphocytes, deficiency of T-helpers as well as increase the contents of T-suppressors were mostly detected. The activation of immune parameters occurs in some patients was due to changes of quantity and increase of activity T-helpers and in others was due decreasing of quantity T-suppressors. Thus, EED methods did not simply change the level of T-lymphocytes, but led to the normalization of the helpers/suppressors ratio. The imbalance of the ratio of T-helpers/T-suppressors could warn against the development of lung autoimmune processes [24].

The initial disorders of immune response in these patients concerned mainly the cellular link. Thus, humoral immune status was characterized by the increase of IgM, IgA, IgG levels. The low level of B-lymphocytes against the background of the high level of Ig reflected a high functional condition of Ig. The significant increase of the levels of IgA, IgG, IgM confirmed the fact, that the immune protection of these patients was formed, mainly, by the humoral immune response.

Complex and heterogeneous pulmonary changes during severe CAP evolution, cause disorders of organs and systems function and demand including into its treatment the new methods which would stimulate the protectively-adaptive reactions and immune response as well as would have disintoxication action and ability to regulate the lipids peroxidation. EED methods can provide these effects.

The fluctuations of Ig levels led to the some normalizing influence on IgA, IgG (less IgM) values. It is possible to regard it as immune-correcting action of EED methods due to the activation of antibodies function and antibodies-formatted cells against the background of an acute lung inflammation.

The additional using of these methods promoted the increasing activity of the immune response by the 12-14th day of therapy with the subsequent tendency to normalization of these parameters against the background of clinical and radiological resolution of severe CAP. We revealed the positive dynamics of clinical and radiological signs in 96%, 91% and 95% of the 2nd, 3rd and 4th patient's groups respectively after finishing the treatment.

Three procedures of UVLE positively influenced the patient's immune response as well as the balance in the system of proteinase/inhibitors and stimulated the reduction of the terms of clinical and radiological resolution of this severe disorder in contrast to the standard management.

The complex positive effect of UVLE could be caused by a heterogeneous action: formation of photoproducts (as a result of loss of molecules of electrons, restructuring of molecules and their disintegration), UV rays action on cell membranes with the subsequent increasing of phagocytic activity, secretion of bactericidal proteins and interleukins; disintoxication and anti-inflammatory actions (direct bactericidal action due to the break of chemical communications and disorders of cell's structures); mediate influence due to the actions of the biologically active substance formed in the cells as well as antioxidant action (due to the stimulation of synthesis of antioxidants and increase the level of trap's substances of reactive oxygen species) and stabilization of PL [26].

The only stay of blood outside of a vascular bed changes its properties (even during short time) and return blood to body is accompanied by the significant biological action. One of mechanisms of UVLE is connected with structurally functional changes of a surface of blood cells and entrance of the near-membrane components in a blood flow. The changes of cell glycocalix are accompanied by modifying of receptors activity of and the antigens which were localized on a cell surface [27,28]. It was complicate for us to verify this UVLE action because it supplemented the standard therapy of severe CAP.

The above mentioned mechanisms could: stimulate the metabolic and regeneration processes, modulating effect during

change of immune status parameters and PL processes; produce bactericidal and anti-inflammatory effects; improve microcirculation and reduce tissues hypoxemia [29-32]. We did not observe the exhaustion of a pool antioxidant defense during UVLE.

Just a single using of BSS significantly decreased the signs of intoxication as well as blood levels of elastase and trypsin-like proteases in severe CAP patients. The using of 3 procedures of BSS normalized some parameters of the immune response and imbalance of PL/antioxidant defense as well as reduced the terms of clinical and radiological resolution of this pathology in contrast to traditional treatment. The including of BSS in management of this pathology treatment provides more influence on the level of protease activity as compared with UVLE.

SOD, as well as catalase, participates in regulation of body oxidizing processes. Thus, the condition of antioxidant defense was characterized by a compensated significantly increasing activity of SOD. Meanwhile, catalase activity was significantly lower when compared with the control one in patients of all investigated groups. Such a reduction of catalase activity against the background increase of MDA should be regarded as the certain exhaustion of antioxidant defense. Similar data (expressed increase of free radical oxidation that was accompanied by the expressed reduction of antioxidant protection in severe CAP patients) was reported by Trubnikov [33]. Recent studies [21,29] have detected the absence of an adequate reaction from antioxidant defense enzymes in this disorder against a background of increase of LP products and reactive oxygen species from inflammatory cells. Decreasing of catalase activity could act as a risk factor of delaying the resolution of severe CAP [34,35]. Probably, the low level of LP products is the adverse prognostic sign in this pathology. The intensity of LP processes is the protective reaction [36] and do not require cessation of this process but only regulate its rate.

The significant increase of SOD activity as well as the small increase of catalase activity against the background of certain decrease of the baseline MDA level were observed in dynamics in patients who were treated with EED methods. The considerable improvement of the immune response in patients of the 2nd-4th groups after these procedures made it possible to consider that the antioxidant defense (supervising PL) makes the condition for the adequate immune response to the influence of the infectious agent. SOD activity and MDA level tended to reduce as well as to increase of catalase activity in patients who were standard treated by the 12-14th day.

The normalization of the imbalance of PL/antioxidant defense was noted in patients who underwent EED methods application by the 20-22th day. The normalization of functional catalase insufficiency was promoted by BBS or BSS plus UVLE additional using. These methods may decrease PL followed by the improvement of the patient's general condition.

The decreasing of the MDA level (the final product of PL) in patients who were treated with the using of EED methods, was testified by a more expressed stabilizing effect of UVLE, BSS and their combination upon the cellular membranes in contrast to the traditional treatment. During the latter a significantly higher MDA level was detected by the 20-22th day. The high level of products of PL as well as antioxidant defense persisted in patients who were standard treated till the moment of their discharge from hospital [29]. MDA, reacting with amino groups of proteins, could change the structure of elastic fibers in pulmonary tissue; break the function of aerohematic barrier as well as the intensity of pneumosclerosis processes during severe CAP [37].

The imbalance in the systems of proteinase/inhibitors as well as in PL/antioxidant defense develops in this pathology against the background increase of the MM level. The latter was seen naturally in this situation. These disorders were not sufficiently eliminated by antibiotics therapy only.

The condition of proteinase system was characterized by a significant increase of the elastase and trypsin-like activity against the background of expressed deficiency of the proteinases inhibitors (α_1-AT and α_2-MG) in severe CAP patients on admission. α_1-AT is the marker of acute inflammation and its level should be significantly increase during this disorder. Granulocytic elastase could cause the degradation of α_1-AT [38] as well as destroy all the basic lung structures (including of bronchi, alveoli and vessels). The low level of α_1-AT could explain by oxidizing denaturation of α_1-AT by various oxidizing agents [39]. The decrease of rate association between enzyme and inhibitor could allow the elastase activity to realize this destructive action during severe CAP [40]. Thus, the increase of serum protease activity in this pathology as well as accumulation of MM against a background of the small enhancement of α_1-AT and α_2-MG levels was reported by Egorshina [21].

Our results showed that the treatment with EED methods is effective in severe CAP with endogenic intoxication syndrome. Thus, a single using of BSS plus UVLE in these patients rendered the expressed positive influence upon the proteinase activity in blood. It was accompanied by the positive clinical dynamics (reduction of fever, weakness, breathlessness, tachycardia as well as an improvement of common health state).

The application of EED methods (better BSS plus UVLE) in CAP therapy is more effective (as compared with standard therapy only) for quicker improvement of the imbalance in oxidant/antioxidant and proteinase/antiproteinase systems. Even a single carrying out BSS and BSS plus UVLE caused the significant decrease of elastase and trypsin-like activity, as well as MM level. The latter is the marker of activation of endogenous proteolysis and the expressiveness of endogenic intoxication syndrome [26]. The high MM level could break microcirculation, reduce of erythropoiesis, stimulate development of a secondary immunodeficiency and reduce the synthesis of proteins as well as the level of tissue respiration [41-43].

The obtained data could be explained by the fact, that during procedures of BSS some exo-/endogenic metabolites from the patient's body were eliminated. Such positive dynamics was accompanied by decrease of the endogenic intoxication signs (reduced BT, HR, RR and CSI). These results were in accord with the concept of the key role of proteolysis hyperactivity in generation of endogenic intoxication syndrome [26].

We observed every other day the repeatedly increasing activity of proteinase, but it did not reach an initial level. It was caused by going out of proteases into a blood according to gradient of concentration against a background the decrease of microcirculation blockade [22]. A single carrying out UVLE did not influence the protease system, however, it was accompanied

by reduction of the endogenic intoxication signs too. The similar clinical benefits of a single application of UVLE were observed by other researchers [29,32,44]. Our results extend those of previous studies by showing greater reduction of endogenic intoxication syndrome by using of EED methods.

We revealed that these methods could provide the significant benefits, largely by reducing this syndrome. Thus, carrying out the BSS or UVLE courses (as their combination) led to the significant decrease of elastase and trypsin-like activity (by about 2 times) as well as MM level and to increase of antiproteinase level (by 2 times) by the 12-14th day. These parameters were subsequently normalized by the 20-22th day of treatment. Meanwhile, in the 2nd group the decrease rate of protease activity was notably lower and by the 20-22th day the high elastase activity level persisted against a background of normalization of other parameters.

Normalization of CSI and the radiological resolution of severe CAP were detected by the finishing treatment in majority of patients (95% of patients of the 3rd-4th groups). The time of this pathology radiological resolution in the 2nd group significantly did not differ from the 3rd and 4th groups.

Some increase of elastase activity against a background of small decrease of trypsin-like activity as well as MM level ($P<0.05$) and several enhancement the level of protease inhibitors was observed during standard therapy by the 12-14th day. The high levels of elastase and trypsin-like activity as well as MM were detected by the 20-22th day in patients who were conventional treated.

Those, who underwent such therapy, received a worse clinical dynamics. The dynamics of protease activity and MM level was accompanied by the later terms of CSI decrease and radiological resolutions, as compared with the patient's groups those were treated with using of EED methods.

Our data showed the favourable outcomes in severe CAP patients who were additionally early treated with EED methods vs conventional management only. Thus, the using of EED methods allowed decrease the length stay in ICU for this disorder in contrast to traditional treatment. The including of UVLE cut down therapy costs of this pathology by 14%, either BSS or BSS+UVLE by 18% in spite of additional application of these methods.

Conclusions

Our results suggest the innovative therapeutic options for severe CAP, which could improve the outcome. This trial has shown the benefit of EED methods including in traditional therapy of this pathology. Thus, EED methods improved the patients' general condition and treatment failure rate.

The introduction of BSS and UVLE into severe CAP therapy has significantly sped up the decrease of elastase and trypsin-like activity, the MM level, simultaneously the increase of α_1-AT and α_2-MG levels in contrast to the standard management. Positive dynamics of these laboratory parameters was accompanied with the significantly faster improvement of the patient's general condition (symptoms disappeared quicker in the 2nd-4th groups by 2-4 days vs the 1st group), the decrease of CSI as well as reduction of CXR terms resolution in comparison with traditional management of this pathology.

Combination of BSS plus UVLE improved treatment efficacy of severe CAP as well as provided the similar effect with single BSS using. But this combination quicker normalized vesicular breath in contrast to standard therapy and single application of BSS or UVLE. These two management strategies (BSS or UVLE) appear equally effective in this disorder treatment.

Thus, the use of EED methods in severe CAP therapy is more effective (as compared with the standard management only) for quicker improvement of patient's status as well as for decreasing the treatment period, which makes EED methods an attractive therapy option.

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The efficacy and safety of argon plasma coagulation (APC) in the management of polyp remnants in stomach and colon

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Abstract

Purpose: Endoscopic treatment of sessile and semipedunculated polyps remains controversial. Residual tissue remains frequently after endoscopic snare polypectomy. The aim of the study was to assess the outcome and safety of argon plasma coagulation (APC) in the management of gastric and colorectal polyp remnants after polypectomy, and to search for clinical parameters useful in predicting the efficacy of this technique.

Material and methods: This prospective study comprised 18 patients with gastric polyps and 29 with colonic polyps found in upper and lower GI endoscopy. Overall 22 gastric polyps and 58 colonic polyps have been detected. All those polyps were removed at colonoscopy with the diathermic snare and the polyp remnants were destroyed with APC using Argon Beamer source (Erbe, Germany). Follow-up endoscopies have been performed 1, 3 and 6 months after the treatment completion.

Results: Pathologic examination revealed 10 hyperplastic polyps and 12 tubular adenomas of the stomach. Effective destruction of polyp remnants was achieved in 20 (90.9%) gastric polyps in 16 (88.9%) patients. Significant positive correlation was demonstrated between the power output, APC sessions number and polyp location in the prepyloric part, its size and adenomatous content. Among colonic polyps there were: 17 hyperplastic, 26 tubular, 8 tubulo-villous, 4 villous adenomas and 3 inflammatory pseudopolyps. Effective destruction of remnant polyp tissue was obtained in 56 (96.4%) polyps in 27 (93.1%) patients. A significant positive correlation between the power output and the size, distal location and villous texture of the polyp has been demonstrated. No complications other than mild abdominal distention have been encountered.

Conclusions: APC is an effective and safe method in the management of polyp remnants in the stomach and colon. The application of higher electric power and numerous APC sessions are necessary to remove residues of large gastric polyps located in the prepyloric part and of with adenomatous content. In the case of colonic polyps the application of higher electric power should be recommended in case of large-sized lesions, located in rectum and of villous texture.

Key words: argon, plasma coagulation, polyp remnants, stomach, colon.

Introduction

Gastrointestinal cancers belong to the most frequently occurring epithelial neoplasms in humans. Their treatment results remain unsatisfactory since in considerable number of cases the surgical treatment is being introduced in advanced cases. Therefore, optimal treatment of precancerous conditions is gaining the significant interest.

Gastric polyps include a number of lesions, among them non-neoplastic polyps, like: hyperplastic and hamartomatic, as well as neoplastic: tubular, tubulo-villous and villous adenomas [1]. Hyperplastic and adenomatous polyps belong to the most frequently occurring. The probability of malignant transformation of gastric adenoma depends on its histologic type, size, macroscopic appearance and the state of underlying mucosa [2]. The presence of adenoma increases the risk of carcinoma development not only within the polyp itself but also in other regions of the stomach [3]. Due to the risk of malignant transformation it is recommended to remove gastric polyps with endoscopic polypectomy [4].

It is widely accepted that nearly all colorectal cancers arise from benign neoplastic polyps [5]. Pathologic evaluations indicate that malignancy rarely occurs in tubular adenomas, and is more common in tubulo-villous and villous adenomas [5]. Based

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on the well-known adenocarcinoma sequence and the malignant potential of adenomas, complete removal of these lesions is indicated [6]. A recent study of postpolypectomy surveillance demonstrated a 66% reduction in colorectal cancer incidence with this procedure [7].

Most pedunculated adenomas can be excised endoscopically with diathermy snares. However, sessile adenomas are less well suited for snare resection.

The management of sessile polyps, small and very big, is the subject of much controversy. The removal of large or sessile lesions with "piecemeal" polypectomy is most frequently not radical and burdened with high risk of complications [8].

In the last decade, argon plasma coagulation (APC) has been introduced in endoscopic treatment of large polyps. This method has been known before and used for a long time in open surgery to control superficial, extensive bleedings from parenchymatous organs. Lack of electrode contact with coagulated tissue, no smoke production and no tissue carbonization contributed to the introduction of this technique to endoscopy in 1993 [9].

In APC the current is transmitted to the tissue through ionized, electrically inert argon gas. It comes to its ionization in high-frequency electric field created between the electrode of the applicator and the tissue. In the course of coagulation it does not come to vaporization ("vanishing") of tissue. The depth of coagulation is automatically limited by thin, insulating layer formed as a result of desiccation of tissue surface [10].

The clinical efficacy, safety and specific indications for APC have not been clearly established. In addition, the correlation between different clinical and APC technical parameters has not been studied yet.

The aim of the study was to evaluate the effectiveness of argon plasma coagulation in destroying remnants of gastric and colonic polyps after endoscopic polypectomy and to search for clinical parameters useful for predicting efficacy of this technique.

Material and methods

This prospective investigation comprised 18 patients with gastric polyps aged 42-72 years (mean 57.7 years) and 29 patients with colonic polyps, aged 47-75 years (mean 59.5 years) treated in Gastroenterology Ward, Regional Hospital in Piotrków Trybunalski, Poland. Those lesions have been diagnosed at upper GI endoscopy and colonoscopy.

Among patients with gastric polyps there were 10 men and 8 women. Fourteen patients had one polyp each and four – two polyps each. Two polyps have been located in the cardia, eight in the body, six in prepyloric part and two – in the postresectional anastomosis area. Overall, APC has been applied in 22 polyps.

In the group of patients with colonic polyps there were 12 men and 17 women. In some of them multiple lesions have been found, therefore APC has been used overall in 58 colonic polyps. Nineteen patients had one polyp each, three – two polyps, four – three polyps, two – four polyps and one – thirteen polyps. The polyp location was the following: rectum – 33 polyps, sigmoid – 13, descending colon – 7, transverse colon – 1, ascending colon – 1 and caecum – 3.

For all the examined subjects individual protocols have been worked out containing the results of preliminary and follow-up clinical, endoscopic, histopathological and imaging data as well as the description of coagulation with argon beamer and its settings.

All polyps have been removed with diathermic snare and all the obtained material, fixed in 10% buffered formalin, was subjected to routine pathologic examination. On obtaining the result of pathologic examination (within 2-7 days), the patients were qualified for coagulation with argon beamer.

There following criteria of qualification for the removal of texture remnants of polyp after endoscopic polypectomy with APC have been established:

1. Sessile or semipedunculated polyp,
2. Diameter 8-20 mm,
3. No traits of severe dysplasia in pathologic examination.

The aim of argon plasma coagulation in the case of gastric and colonic polyps was the total destruction of polyp remnants. We arbitrarily assumed this method to be satisfactory, if this effect is obtained at least in 80% of cases.

Informed consent has been obtained from all patients, and the study protocol was approved by the local Ethical Committee at Medical University of Łódź, Poland.

Argon plasma coagulation was performed with the ERBE Argon Beamer source (Erbe, Germany) containing Erbotom ICC 200, Argon Beamer 2 and flexible applying catheter. After exposing the lesion at endoscopy and introducing the APC probe the procedure has been performed. In case of gastric polyps the power output was initially set at 50-80 W, in case of proximal colon lesions – at 40 W, and in distal colon – at 60-80 W. In case of visibly insufficient coagulation it has been increased gradually by 5 W up to 60 W – in the proximal colon, 80 W – in the stomach and 90 W – in the distal colon.

The APC sessions number ranged from 1 to 7 in one patient, with 2-4 days intervals, until total destruction of residual tissue has been achieved.

First follow-up endoscopy has been performed 2 days after coagulation and the next clinical and endoscopic examinations were carried out after 1, 3 and 6 months following the treatment completion. In each patient, video documentation of performed endoscopic procedures has been recorded in order to identify the sites after polypectomy and earlier coagulation.

Statistical analysis was performed using method of linear regression and Student's t-test for unpaired results. Determination coefficient (R^2) and Durbin-Watson statistic value (DW) has been calculated. Differences were considered to be significant at $p<0.05$.

Results

Gastric polyps

Pathologic examination revealed 10 hyperplastic polyps and 12 tubular gastric adenomas.

In all cases a partial polypectomy with diathermic snare has been performed and the residues have been destroyed with APC. The patients underwent 1 to 3 APC sessions (mean 2.5). The applied coagulation energy ranged from initial 50 W to 80

Table 1. **Clinical data referring to APC procedure in gastric and colonic polyps**

	Gastric polyps	Colonic polyps
Size of polyps	8-20 mm	8-20 mm
Number of sessions	1-3 (mean 2.5)	1-7 (mean 1.7)
Intervals between sessions	2 days	2-30 days
Applied energy	50-80 W	40-80 W
Complete treatment time	1-6 days	1 day-2 months
Follow-up complications	None	None
Effectiveness	20 polyps (90.91%)	56 polyps (96.55%)
Therapeutic failures	2 polyps (9.09%)	2 polyps (3.45%)

Figure 1. **The relation of electric power output and gastric polyp location**

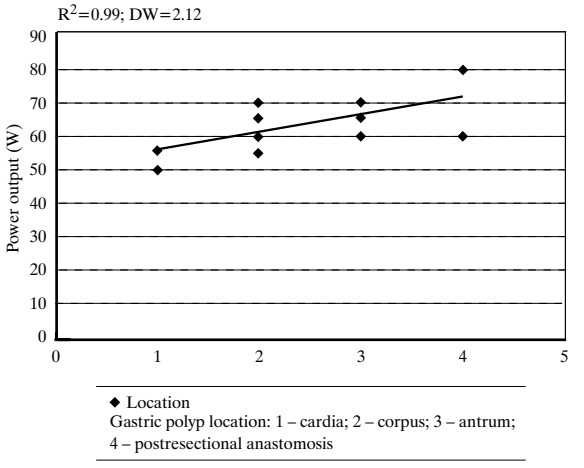


Figure 2. **The correlation of electric power output and the gastric polyp size**

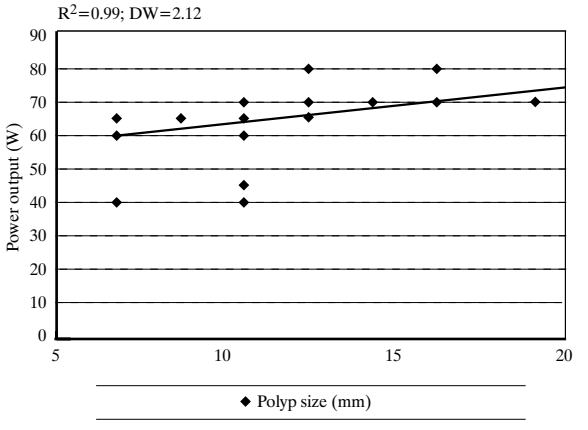


Figure 3. **The correlation of electric power output and the gastric histopathology**

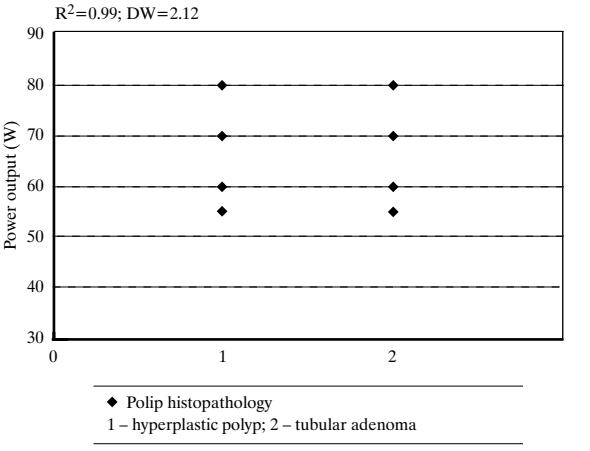
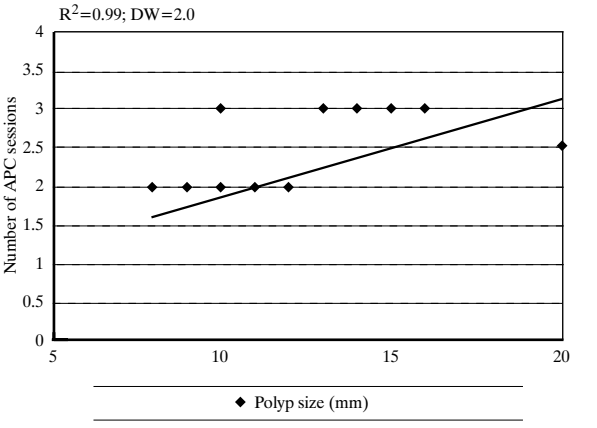


Figure 4. **The correlation between the number of APC sessions and the gastric polyp size**



W. Four polyps' remnants have been destroyed in the course of one session, 12 – in two and 6 – in three sessions. In 20 polyps (90.9%) in 16 patients (88.9%) complete destruction of remnant tissue has been revealed at the second control gastroscopy, one month after APC treatment (*Tab. 1*). In this group, no recurrences have been noted until 3 years after the procedure. Therefore, the obtained results of APC application have been better than the intended 80%. In two cases the follow-up endoscopy one month after APC completion, revealed the polyp recurrence. They were both hyperplastic polyps with the diameter of 8 mm and 10 mm, located in the postresectional anastomosis area.

Significant positive correlation has been demonstrated between the final power output used and the location of the polyp in prepyloric part, its larger size and adenomatous morphology (in all cases: R^2=0.99; DW=2.12; *Fig. 1*, *2*, *3*). Significant positive correlation has also been found between the number of APC sessions and the size of polyp (*Fig. 4*; R^2=0.94; DW=2.0), adenomatous morphology (R^2=0.82; DW=1.3) and the location in prepyloric part of the stomach (R^2=0.84; DW=0.82).

In all patients in the course of APC procedure, a sensation of mild distension in epigastrium, disappearing after the withdrawal of air and argon from the stomach has been observed. No severe complications, such as perforation of the stomach wall have been encountered.

Colonic polyps

Among colonic polyps pathologic examination revealed: 17 hyperplastic polyps, 26 tubular adenomas, 8 tubulo-

Figure 5. **The correlation of electric power output and the colonic polyp size**

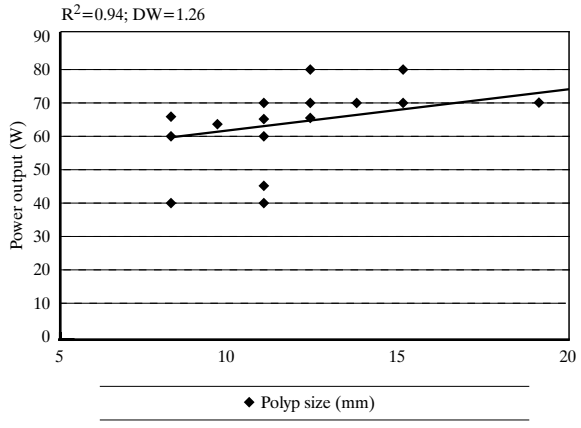
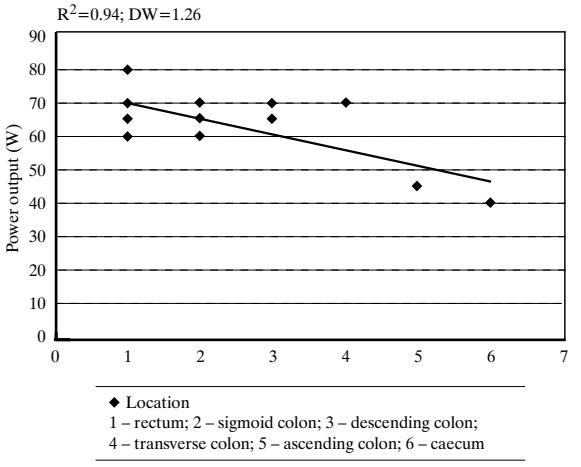


Figure 6. **The correlation of electric power output and the colonic polyp location**



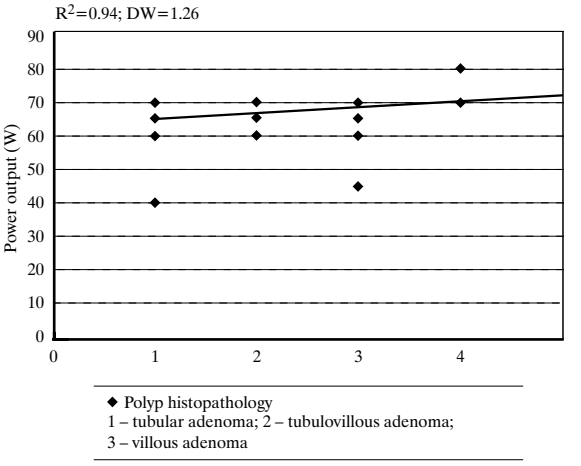
-villous adenomas, 4 villous adenomas and 3 inflammatory pseudopolyps. In all cases partial polypectomy with diathermic snare has been performed and the residue tissue destroyed with APC, which was carried out in 1-7 sessions (mean 1.7), in 2-4 days intervals. Power output of 40-50 W was applied for polyps located in caecum, 45-55 W – in ascending colon and 60-80 W for polyps of transverse, descending, sigmoid colon as well as the rectum (*Tab. 1*).

Complete destruction of the polyp remnants, observed also in all successive follow-up colonoscopies has been achieved in case of 56 polyps (96.4%) in 27 patients (93.1%). Among them, 39 polyps were destroyed in the course of one session, 8 – in two, 7 – in three, 2 – in four and 2 – in 7 sessions. In two patients with single villous polyps, despite initial successful remnants destruction, follow-up examination after 3 (in first case) and 6 months (in the second one) revealed single villous polyp in the postpolypectomy site.

Significant positive correlation has been demonstrated between the final power output used and the polyp size and its distal location within colon (in both cases: R^2=0.94; DW=1.26; *Fig. 5*, *6*). In addition, the power output was the highest in case of villous adenomas, lower – in tubulo-villous and the lowest – in tubular adenomas and hyperplastic polyps (*Fig. 7*; R^2=0.94; DW=1.26).

In the course of APC sessions, 16 patients (55.2%) complained of flatulence. No other symptoms or complications have been observed.

Figure 7. **The correlation of electric power output and the colonic polyp histopathology**



Discussion

In the last 25 years endoscopic polypectomy has become the procedure of choice in pedunculated polyps of the digestive tract. However, no uniform standards of managing polyps of large sizes, non-pedunculated and situated in thin-walled organs have been established yet. Therefore a great interest is raised by new techniques which potentially could be useful in such cases.

In the presented study the complete destruction of 20 out of 22 gastric polyps (90.2% of the cases) has been achieved. Thus, the efficacy of the presented method in eradication of residues after polypectomy of gastric polyps should be estimated as very high. APC treatment appeared to be ineffective for two hyperplastic polyps, of 8-10 mm diameter, located in the anastomosis area after partial gastrectomy. This should be explained with the increased mucosal proliferative activity, frequent development of atrophic gastritis, intestinal hyperplasia and metaplasia in this area [11].

In our study the final current power applied in APC depended on the size of gastric polyp and its histopathological parameters. Large adenomatous polyps required higher electric power than smaller, hyperplastic ones.

In addition, in case of large polyps confining to one treatment session was usually insufficient and more APC sessions were necessary. Up to now, to our knowledge, no similar analyses have been reported.

In the presented study argon plasma coagulation has been applied to eradicate 58 colonic polyps remnants after endoscopic polypectomy. Complete destruction of those remnants,

with no recurrences in the follow-up period up to 6 months, was achieved in as much as 56 (96.55%) of them. Therefore, those results should be considered as highly satisfying.

Grund, Storek, and Farin, as well used APC to remove residues of colonic adenomas. They subjected 13 patients to 19 sessions of APC, achieving in all cases the destruction of adenomas, enduring 1-72 months' of follow-up period [9]. Regula et al. used argon plasma coagulation to eradicate remnants of large sessile colorectal polyps after snare piecemeal polypectomy in 63 patients [12]. In follow-up examinations carried out in the mean period of 37 months after the treatment, recurrent adenomas has been found only in 14% patients Similarly, Garcia et al., showed the successful APC ablation of colorectal polyp remnants in 90.9% of adenomas, with 20% recurrence rate during mean follow-up period of 16.3 months [13]. All of the recurrent polyps have been located in the rectum, which corresponds with our observation on the need of using higher power output for distal colon polyps. In the randomized study, even in patients after complete endoscopic snare resection of large adenomas, APC significantly reduced adenoma recurrence [14]. However, Zlatanic et al., applying APC in a similar case in 30 patients observed recurrences of polyp in as many as 50% of the cases during five and a half months' follow-up period [15]. The fact that in this study almost all polyps treated with APC were tubulo-villous adenomas might explain this high number of recurrences. In the presented study, the follow-up examinations carried out 3 and 12 months after the last APC session, only in 2 cases (3.45%) showed a polyp recurrence, which in both cases were villous adenomas.

On the other hand, Wahab et al. used APC to destroy residues of villous adenomas in 28 patients, achieving recovery in as many as 100% during 3-18 months' follow-up period [16]. Good results presented by these authors are most probably related to the high number of performed sessions (10-13) and longer treatment (up to 13 months). However, it should be emphasized that both, our own results and the reports of many other authors show the high clinical aggressiveness of adenomas with villous content. Growth kinetics studies revealed the increased proliferative activity and the extension of proliferative zone in villous adenomas, compared to tubular and tubulo-villous adenomas [17]. High number of recurrences of villous polyps in the same place has also been observed after surgical polyp removal [18]. It has been proven that patients with the history of villous adenomas are at increased risk for advanced adenoma and colorectal cancer at follow-up and are therefore recommended to undergo colonoscopy 3 years after their polypectomy, compared to 5 years for tubular adenomas [19]. It seems, that those observation indicate, the need for more careful monitoring and management of villous lesions, compared to other adenomas.

Another important clinical problem is polypectomy safety in case of their location in thin walled organs, as caecum and ascending colon, where the risk of perforation is high. The application of laser thermoablation in this case may increase the risk of perforation, and furthermore it is difficult, considering the necessity to immobilize the endoscope which precludes any changes in its position during the procedure [20]. The use of APC seems to be an advantageous solution in these cases because on principle it is a method of definite coagulation of shallow depth and the application of low electric power is the additional protection against perforation. In the presented study 5 polyps of caecum and ascending colon were subjected to APC with no further complications.

In the presented study no serious complications following APC have been observed. Similarly, in the comprehensive work summing up 1606 cases of APC application for various indications, Grund and Farin indicated the high safety profile of this procedure [21]. In the study comprising 697 subjects, only in 0.31% of them the perforation occurred. Intestinal wall emphysema has been observed in 0.5% patients and mortality – in 0.14%. Other authors confirm particularly rare occurrence of complications with the use of this method [13,14,22]. It should also be emphasized that in the course of APC therapy practically does not exist a risk of bleeding from gastrointestinal tract, which is confirmed by our experiences. This method has already gained an unquestioned position in the treatment of bleedings from alimentary tract angiodysplasia, where it is being recommended as "the gold standard", as well as in the course of radiation-induced intestinal injury.

To sum up, it should be stated that argon plasma coagulation is an extremely effective and safe method of removing remnants after endoscopic polypectomy performed in the gastric and colonic lumen. The application of higher electric power and numerous APC sessions are necessary to remove residues of large gastric polyps located in the prepyloric part and with adenomatous content. In case of colonic polyps the application of higher electric power should be recommended in case of large-sized lesions, located in rectum and with villous content.

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Plasma adiponectin and E-selectin concentrations in patients with coronary heart disease and newly diagnosed disturbances of glucose metabolism

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Abstract

Purpose: Adiponectin is a fat derived hormone, which enhances insulin sensitivity. In experimental studies adiponectin was shown to have antiatherogenic properties by suppressing endothelial expression of adhesion molecules. Therefore, the aim of the study was to evaluate plasma adiponectin and E-selectin concentrations in patients with coronary artery disease and impaired glucose metabolism and evaluation of their relationship with selected anthropometric, biochemical and clinical parameters.

Material and methods: The study group consisted of 62 patients with coronary heart disease, without previous diagnosis of diabetes mellitus (mean age 48.6±6.0 years; mean BMI 28.6±3.13 kg/m^2). In the studied group the OGTT with glucose and insulin estimation was performed and insulin resistance index (HOMA-IR) was calculated. In the fasting state, the plasma adiponectin, soluble form of E-selectin, HbA1c and lipid parameters were estimated.

Results: Adiponectin concentration was not different in patients with type 2 diabetes mellitus and impaired glucose tolerance (n=36) in comparison to the group with normal glucose tolerance (n=26). There was also no difference in adiponectin concentration in relation to atherosclerosis progression. There was no significant correlation between adiponectin and calculated insulin resistance index, while there was marked inverse correlation between adiponectin and BMI (r=-0.30; p=0.018), body weight (r=-0.33; p=0.008), E-selectin (r=-0.263; p=0.039), TG concentration (r=-0.27; p=0.036), duration of coronary heart disease (r=-0.33; p=0.009) and borderline significance with ejection fraction (r=-0.268; p=0.06).

Conclusions: Our study supports the hypothesis that adiponectin could be recognised as a protective protein for the development of atherosclerosis.

Key words: adiponectin, E-selectin, coronary heart disease.

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Introduction

Adiponectin is a protein of 30kD, synthesized predominantly by adipose tissue [1]. In recent years, a function of adiponectin was associated mainly with the insulin resistance. The data provided showed, that adiponectin concentration positively correlated with insulin sensitivity measured by the euglycemic clamp, as well as with the activity of insulin receptor [2]. Also, adiponectin function is connected with lipid metabolism and dyslipidemia. It was shown that adiponectin is negatively related to HDL-cholesterol and positively to triglycerides (TG) concentration [3]. Low adiponectin concentration was observed in obesity [4,5], type 2 diabetes [3], coronary heart disease (CHD) [6], and in hypertension [7]. In addition, the experimental studies showed, that adiponectin had not only metabolic, but also antiatherogenic and antiinflammatory effect [8,9]. There was observed, that adiponectin inhibited atherogenesis probably by a influence on the proinflammatory cytokine secretion and by a inhibition of the endothelial expression of adhesion molecules [9].

Epidemiological studies show, that cardiovascular diseases are the main cause of premature mortality among type 2 diabetic patients [10]. In type 2 diabetes the accelerated atherogenesis is observed and diabetes per se is considered as the independent risk factor of CHD [11]. Our previous studies indicated, that in obese patients, with normal glucose metabolism, the concentration of sICAM-1 was significantly increased and correlated with the insulin resistance and with concentration of proinflammatory cytokines, like tumor necrosis factor-α (TNF-α) [12]. Furthermore, in patients with type 2 diabetes or impaired glucose tolerance (IGT) and CHD the increased concentration

***Table 1*. The clinical characteristics of studied group with coronary heart disease (CHD)**

	N (%)
Age (years)	48.6 (±6.0)
BMI (kg/m^2)	28.6 (±3.13)
Hypertension	21 (33%)
History of myocardial infarction	37 (57%)
Family history of CHD	32 (52%)
Smoking	32 (52%)

of E-selectin and VCAM-1 concentration was also noticed [13]. There are several studies concerning adiponectin concentrations in patients with type 2 diabetes mellitus and CHD. For instance, low adiponectin concentration was observed in type 2 diabetic patients with CHD in Japanese population [3].

The aim of the present study was the evaluation of adiponectin concentration in patients with newly diagnosed type 2 diabetes mellitus or IGT and CHD in comparison to the group without disturbances of glucose metabolism. We also aimed to asses the relationship between adiponectin concentration and E-selectin and selected anthropometric, biochemical and clinical parameters.

Material and methods

Sixty two male patients, without history of type 2 diabetes mellitus, with stable CHD were recruited for the study. The patient was considered to have CHD if he had history of myocardial infarction or the diagnosis was based on the result of coronary angiography. In the studied group 57% of the patients had history of myocardial infarction, 52% were smokers. Clinical characteristic of the study population is presented in *Tab. 1*. The consent for the study was obtained from all participants, and the protocol was approved by Ethics Committee of Medical University in Białystok.

In all subjects the standard oral glucose tolerance test (OGTT) after 75 g oral glucose was performed. Glucose and insulin concentrations were measured at 0 min, 60 min and 120 min during the OGTT. Type 2 diabetes mellitus or IGT was diagnosed in accordance of the WHO guidelines from 1999. Plasma glucose was measured immediately by the oxidase method on automatic glucose analyzer (YSI START PLUS 2300) and samples for plasma insulin estimation were frozen in -20°C, until assayed. Plasma insulin concentration was measured using IRMA method (Polatom, Świerk, Poland).

Before the OGTT, blood samples were drawn also for plasma measurements of adiponectin (RIA, Linco Research Inc., USA), selectin E (ELISA, R&D System, USA), HbA1c (HPLC-BIO-RAD, Germany), cholesterol, HDL-cholesterol and TG concentrations (enzymatic method-ANALCO-CBG, Poland). Insulin sensitivity was assessed by indirect indexes: insulin resistance index – HOMA-IR (Homeostasis Model Assessments) based on fasting glucose and insulin concentration [14] and by oral glucose tolerance (IS-OGTT) test according to Matsuda and De Fronzo [15].

***Table 2*. Plasma glucose and insulin concentrations during oral glucose tolerance test and indices of insulin resistance in studied patients**

	Patients with type 2 diabetes and IGT n=36	Patients with normal glucose tolerance n=26
Glucose 0 min (mg/dl)	95.2±14.9	85.6±10.6*
Glucose 60 min (mg/dl)	193.1±41.0	139.0±30.4*
Glucose 120 min (mg/dl)	173.5±33.5	107.5±19.6*
Insulin 0 min (mU/l)	11.6± 8.09	8.9±8.6
Insulin 60 min (mU/l)	85.3±82.0	106.1±68.0
Insulin 120 min (mU/l)	92.4±82.4	48.0±46.4 p=0.05
HOMA-IR	2.04±2.36	2.76±2.00
IS-OGTT	4.22±2.4	8.13±5.02*

*p<0.05 patients with type 2 diabetes and IGT in comparison to the group with normal glucose tolerance

Statistical analysis was performed by using STATISTICA Stat-Soft 5.0 program. The comparison of data between the groups was done with the Student t-test. To analyze factors correlated with adiponectin, Pearson's correlation index was used.

Results

In the studied group, 6 of the patients were diagnosed to have type 2 diabetes and 30 patients to have IGT, basing on the results of the oral glucose tolerance test. In whole group, 58% of patients presented disturbances of glucose metabolism. Plasma glucose concentrations during OGTT was statistically significantly higher among patients with disturbances of glucose metabolism (0 min; $p<0.01$, 60 min; $p<0.001$, 120 min; $p<0.001$) (*Tab. 2*). Also, the insulin sensitivity index calculated from the plasma glucose and plasma insulin level during oral glucose tolerance test was significantly lower in this group ($p=0.0004$) (*Tab. 2*). HOMA-IR did not differ between studied groups.

Plasma adiponectin concentrations in patients with diabetes and IGT were not significantly different compared with patients with normal glucose tolerance (*Tab. 3*). Also, when the groups with type 2 diabetes and IGT were considered separately, there were no differences in adiponectin concentration between examined groups (type 2 diabetes – 5.41±0.38 μg/ml, IGT – 6.01±0.81 μg/ml, patients with normal glucose tolerance – 6.22±0.86 μg/ml). There was also no difference in adiponectin concentration in relation to atherosclerosis progression. However, the group of patients with disturbances of glucose metabolism had significantly higher BMI ($p=0.01$), (*Tab. 3*).

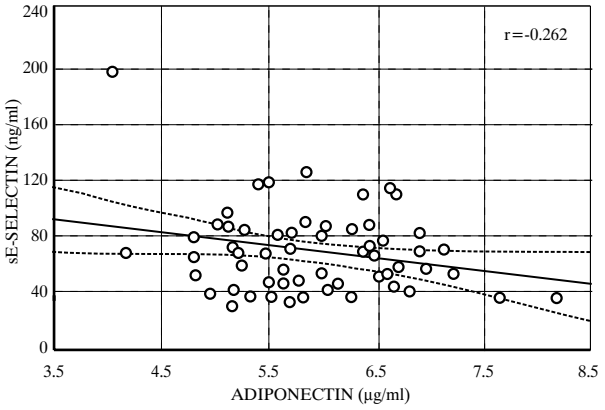
E-selectin concentration was significantly higher in patients with type 2 diabetes mellitus and IGT ($p=0.014$) (*Tab. 3*). There were negative statistically significant correlations between plasma adiponectin level and BMI ($r=-0.30$; $p=0.018$), E-selectin level ($r=-0.26$; $p=0.039$) (*Fig. 1*), duration of ischemic heart disease ($r=-0.33$, $p=0.009$), TG concentration ($r=-0.27$; $p=0.036$) and of borderline significance with ejection fraction ($r=-0.27$; $p=0.06$).

Table 3. **Adiponectin, E-selectin plasma concentrations and selected clinical and metabolic parameters in studied group**

	Patients with type 2 diabetes and IGT n=36	Patients with normal glucose tolerance n=26
Adiponectin (μg/ml)	6.0±0.86	5.91±0.78
E-selectin (ng/ml)	76.2±32.4	58.4±22.3*
Age (years)	47.0±5.75	46.6±6.45
BMI (kg/m^2)	29.5±2.88	27.5±3.2*
HbA1c (%)	5.6±0.49	5.36±0.56
Total cholesterol (mg/dl)	195.6±39.4	210.7±27.1
LDL cholesterol (mg/dl)	127.0±34.7	140.8±24.9
HDL cholesterol (mg/dl)	32.6±11.3	33.5±12.7
TG (mg/dl)	179.2±100.8	177.2±96.0

*p<0.05 patients with type 2 diabetes and IGT in comparison to the group with normal glucose tolerance

Figure 1. **Correlation between plasma adiponectin and E-selectin concentration in studied groups**



Discussion

In our observation, we found IGT in more than 50% of patients with CHD, without previous history of type 2 diabetes mellitus. Similar results were obtained in our study with large cohort of patients with CHD [16], what supports the notion that the disturbances of glucose metabolism are considered to be an independent risk factor in the arteriosclerosis development. In the present study we did not observe any differences of adiponectin concentrations between group of male patients with impaired glucose metabolism and CHD in comparison to the group with normal glucose tolerance. It should be pointed out, that in the present study the comparison was done among group of male patients with CHD according to appearance of impaired glucose tolerance. Hotta et al. showed statistically significant lower plasma adiponectin level in patients with type 2 diabetes and CHD, than in patients with diabetes without CHD [3]. We also noticed that plasma adiponectin concentration was also lower in patients with diabetes, but probably because of the small number of patients with type 2 diabetes (n=6), this association did not reach statistical significance (p=0.11). Yaturu et al. found a statistically significant decrease in adiponectin level in the patients with prediabetes defined as a IGT, and the patients with type 2 diabetes in comparison to the controls [17]. Furthermore, in the prospective study of 745 male patients with type 2 diabetes, high plasma adiponectin level was associated with a significantly lower risk of the cardiovascular events in 5-years observation [18]. Authors suggested that the increased adiponectin levels are associated with a moderately decreased CHD risk in the diabetic men. In contrast, the results from British Women's Heart and Health Study did not show the association of adiponectin with future risk of CHD in women [19]. However, other investigators, found a lower adiponectin concentration in the patients with CHD comparing to control group, in groups of patients without diabetes [5,20]. The latest data showed, that also in type 1 diabetes, the higher adiponectin level was associated with a lower risk of CHD [21]. The review of the data showed the connection of hypoadiponectinaemia and CHD [3,5,17,18,20]. Additionally, in this study we also observed the negative correlation between the plasma adiponectin concentration and duration of CHD.

The next question, which needs an explanation is a mechanism of the adiponectin antiatherogenic effect. It is known, that adiponectin is the protein synthesized predominantly by adipose tissue and plays an important role in the glucose and lipids metabolism [22]. As we mentioned at the beginning, adiponectin function is connected mainly with insulin sensitivity. In recent years more attention is paid to adiponectin influence on blood vessels. Studies *in vivo* and *in vitro* showed, that adiponectin concentration correlated with vasodilatation and that this effect was independent of insulin [23,24]. Studies *in vitro* also indicated, that adiponectin had anti-inflammatory effect. Adiponectin inhibits TNF-α stimulated expression of adhesion molecules on endothelial cells and thus prevents the first stages of the development of atherosclerosis [8,25]. In the present study the concentration of soluble form of E-selectin in plasma was measured in all the patients, and it was statistically significantly higher in the group with disturbances of glucose metabolism. Moreover, the investigated adhesion molecule negatively correlated with adiponectin concentration.

In the performed evaluation we observed also a correlation between plasma adiponectin concentration and ejection fraction, of borderline statistical significance. In the previous studies there are only few information about relationship between adiponectin and ejection fraction. In one of the studies, Huang et al. examined the association between those parameters, and they did not observe any correlation between adiponectin and ejection fraction. However, it should be noticed, that they evaluated different group of patients with chronic renal insufficiency treated by peritoneal dialysis and hemodialysis [26].

The obtained results are consistent with clinical data and identify the adiponectin as an important factor, preventing cardiovascular diseases.

Conclusions

Our study supports the hypothesis that adiponectin is a protein with antiatherogenic activities.

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Acute biliary pancreatitis in the era of minimally invasive surgery

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Abstract

Purpose: Opinions about early endoscopic sphincterotomy and time of laparoscopic cholecystectomy in acute biliary pancreatitis are still controversial. Some authors reserved this procedure only for cases in which the stones were visualized during ERCP or patients had clinical symptoms of acute cholangitis. The aim was the assessment of the dynamic of changes of proinflammatory cytokines and white blood cells in time in patients with acute biliary pancreatitis after performed endoscopic sphincterotomy and laparoscopic cholecystectomy.

Material and methods: We enrolled 43 consecutive patients with clinically diagnosed mild forms of acute biliary pancreatitis. All were treated by early endoscopic sphincterotomy and laparoscopic cholecystectomy performed during the first 48 hours after admission.

The course of the disease was monitored by measurement of the level of proinflammatory cytokines.

Results: Marked decrease of the level of proinflammatory interleukins within 24 hours after endoscopic sphincterotomy was observed. Mean values of IL-6 and IL-8 were statistically lower immidiately after this procedure ($p<0.001$). Subsequent decrease was achieved after laparoscopic cholecystectomy. The mean values of TNF-α and IL-12p40 were relatively constant throughout the study period.

Conclusion: All patients suffering from mild acute biliary pancreatitis should be treated by using minimally invasive procedures. However, such a only treatment should be reserved for experienced centers.

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Key words: pancreatitis, surgical procedures, minimally invasive, endoscopy, interleukins, TNF-α, leukocytes.

Introduction

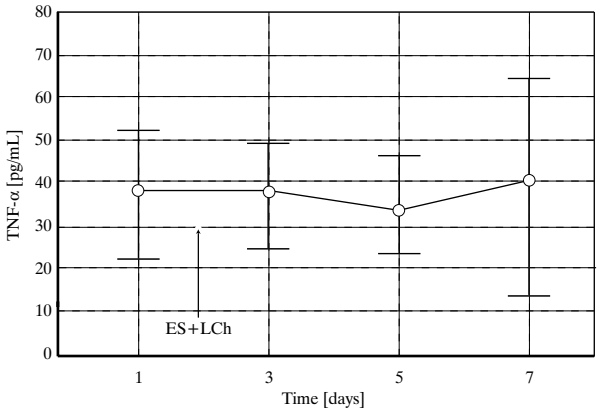
Biliary stones are one of the most important etiological factors of acute pancreatitis. Impairment of bile flow by concrements is the most important mechanism in the development of this disease. The most dangerous stones, called microlithiasis, are 2-3 mm in diameter. Such small particles can easily migrate from the gallbladder to the biliary ducts and also along the biliary tree, consequently blocking the bile flow at the level of the papilla of Vater [1-5]. It was proved that these small stones spontaneously migrate to the duodenum during 48 h and in this period the risk of blockade of the papilla is the highest [4,6]. This migration results in oedema of the papilla of Vater, which is also an important factor in the blockade of bile flow [7]. The "two phases theory" proposes that in the first phase small migrating stones initiate the mild form of acute pancreatitis. In the second phase, persisting stones or repeated passage of small stones effected intermitted or continuous obstruction of the main bile duct and pancreatic duct and developed severe form of the disease [8,9]. It is known, from the experimental and clinical studies, that the time of impediment of bile flow and the possibility of recurrent blockade of the papilla play fundamental roles in the progression of changes in the pancreas [10]. From these reasons is rationale perform early biliary decompression in patients with acute gallstone pancreatitis.

The results of these studies have important clinical implications. The opinions about early endoscopic papillotomy are still controversial. Some authors reserved this procedure only for cases in which the stones were visualized during endoscopic retrograde cholangiopancreatography (ERCP) or when patients had clinical symptoms of acute cholangitis [7,11-13]. However, there were reports suggesting that early improvement of bile flow could reduce the mortality and improve the prognosis in patients with acute pancreatitis [14-20].

Today, the standard of practice requires the removal of the gallbladder during the same hospitalization, but even in the leading centers the time of cholecystectomy is delayed [21].

Taking into consideration the controversy regarding the treatment of patients with biliary pancreatitis and the unpredictable clinical course of the disease, we introduced, as a standard, the early endoscopic sphincterotomy and laparoscopic cholecystectomy. These procedures were performed during the first 48 hours. In this way, we removed the reservoir of biliary stones. These procedures were used in all patients with mild form of acute pancreatitis. To evaluate this model of management of patients with biliary pancreatitis we measured the level of cytokines and absolute account of white blood system during the first seven days of hospitalization.

Figure 1. **TNF-α dynamic changes in time in study group. ES – endoscopic sphincterotomy, LCh – laparoscopic cholecystectomy**



Material and methods

The study was performed on 43 consecutive patients with clinically diagnosed mild form of acute biliary pancreatitis. The diagnosis was based on the clinical picture, ultrasound examination and the increase of the level of amylase in the serum and urine (at least three times above the upper normal limit). The aetiology of pancreatitis was based on the history and ultrasonografic examination which revealed the presence of stones in the gallbladder and/or in the biliary tree. The degree of involvement of pancreas was based on Becker's scale [22]. The increase in size of whole or part of the pancreas with unchanged borders and with diffuse or focal decrease of its echogenicity was classified as 1st degree according to Backer's scale (21 patients). The changes which were characterised by significant increase in the size of the pancreas with the foci of hypoechogenicity in the pancreas and the surrounding fat but with the unchanged border of the pancreas and with the presence of fluid around its nearest neighbourhood were classified as 2nd degree according to Backer scale (22 patients). The clinical severity of the disease was measured using the Apache II scale [23], Ranson criteria [24] and Atlanta criteria [25].

Every patient had endoscopic sphincterotomy with removal of the stones or biliary sludge from the main biliary duct (spontaneously or using the Dormia basket) during the first 24 h after admission. In this way the bile flow was improved. Considering the fact that stones can migrate during the next day, the early cholecystectomy was performed (during the first 48 hours after admission).

During the 1st, 3rd, 5th and 7th day of hospitalization the following biochemical measures were performed: hematocrit, WBC with blood smear, and the level of cytokines such as: TNF-α, IL-1β, IL-6, IL-8 and IL-12p40. The cytokines level was measured using ELISA method (Bio Source Europe SA).

The aim of this study was assessment of the role of endoscopic sphincterotomy on the clinical course of mild form of acute biliary pancreatitis. To achieve this goal we checked the dynamic of changes of proinflammatory interleukins and white blood cells in time in patients with biliary pancreatitis after performed endoscopic sphincterotomy. Also the correlation between the level of interleukins and leukocytes in the 1st day of hospitalization was checked.

Before the statistical analysis was done we assumed that the result "zero" of the concentration of interleukins is caused by the limitation of the used measurement method and not by the real absence of these proteins in the blood. The "zero" were replaced with the minimal results achieved in the whole group of the patients. The results were as follows: IL-1β – 0.59, TNF-α – 0.31, IL-6 – 3.28, IL-8 – 1.88, IL-12p40 – 0.41. Such data were analysed used the model of one factorial analysis of variances for repeated measurements. The impact of endoscopic papillotomy was checked using so-called planned comparison of the contrast method. The mean level of interleukins and leucocytes before the endoscopic sphincterotomy and in the next three measurements after the procedure was compared (contrast: –3, 1, 1, 1). The study of the dynamic of suspected results was based on the analysis of the linear trends (contrast: -3, -1, 1, 3) or if the dynamic of changes had a curvilinear shape based on the square trends (-1, 1, 1, -1). The statistical analysis was done using module GLM of the STATISTICA 6.0 program (company Stat Soft).

Results

In each patient the presence of stones in the biliary tree was confirmed. In 5 patients the stones were not visualized during ERCP, but after sphincterotomy their spontaneous removal was observed. There were no complications observed in any of the patients related to using therapeutic procedures (endoscopic sphincterotomy and laparoscopic cholecystectomy).

The cytokines level

There were no differences between the mean concentration of TNF-α in the following days of the study (*Fig. 1*), ($F_{1,10}$= 0.09; p=0.9610). Also the endoscopic sphincterotomy has no influence on the concentration of TNF-α in the patients' serum ($F_{1,10}$=0.09; p=0.9169).

There were changes of mean concentration of IL-6 after endoscopic sphincterotomy. The level of this interleukin decreased, although this drop was on the border of statistical significance (*Fig. 2*), ($F_{1,18}$=4.37; p=0.0509). The test of the

Figure 2. **IL-6 dynamic changes in time in study group. ES – endoscopic sphincterotomy, LCh – laparoscopic cholecystectomy**

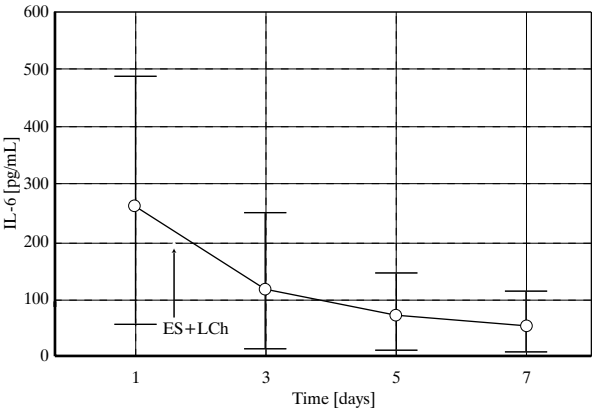


Figure 3. **IL-8 dynamic change in time in study group. ES – endoscopic sphincterotomy, LCh – laparoscopic cholecystectomy**

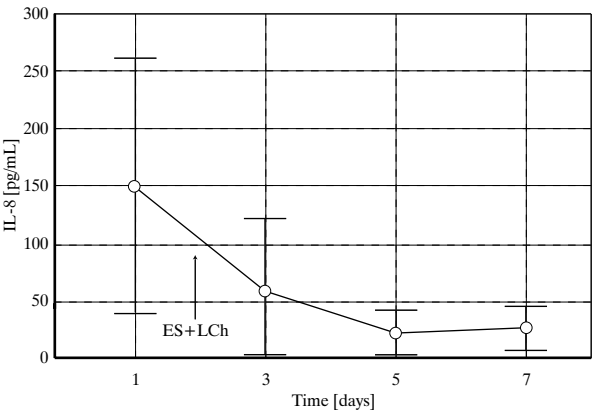


Figure 4. **IL-12p40 dynamic changes in time in study group. ES – endoscopic sphincterotomy, LCh – laparoscopic cholecystectomy**

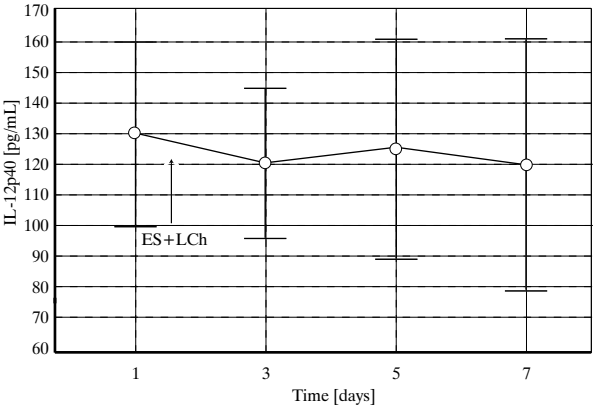


Figure 5. **White blood cells (WBC) dynamic changes in time in study group. ES – endoscopic sphincterotomy, LCh – laparoscopic cholecystectomy**

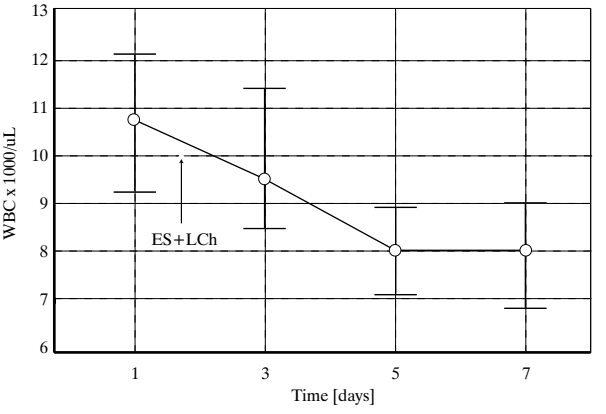
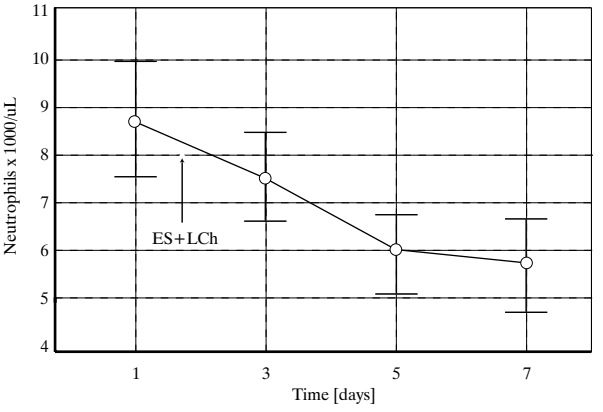


Figure 6. **Neutrophils dynamics changes in time in study group. ES – endoscopic sphincterotomy, LCh – laparoscopic cholecystectomy**



characteristic of trends does not confirm linear other square nature of this process (linear trend – $F_{1,18}$=3.04; p=0.0983, square trend – $F_{1,18}$=2.96; p=0.1021).

The differences of the mean concentrations of IL-8 were statistically significant (*Fig. 3*), ($F_{1,25}$=4.40; p=0.0065). The decrease of IL-8 after endoscopic sphincterotomy was observed ($F_{1,25}$=6.14; p=0.0203). This trend had an asymptomatic character (square trend, $F_{1,25}$=5.04; p=0.0338).

There was no influence of endoscopic sphincterotomy on the concentration of IL-12p40. The mean concentration was on this same level during 7 days of observation (*Fig. 4*), ($F_{1,37}$=0.22; p=0.8806).

The white blood cells system

After endoscopic sphincterotomy the decrease of mean values of leucocytes was observed. This decrease was statistically significant (*Fig. 5*), ($F_{1,38}$=14.42; p=0.0001). The test of the significance of trends showed the linear character of this process ($F_{1,38}$=25.21; p<0.0001).

The mean level of neutrophils also decreased after endoscopic sphincterotomy. This drop was also statistically significant (*Fig. 6*), ($F_{1,38}$=19.94; p<0.0001). The drop had also linear character ($F_{1,38}$=36.32; p<0.0001). No statistically significant changes were observed in the levels of monocytes and lymphocytes after performed endoscopic sphincterotomy (*Fig. 7* and *8* respectively), (lymphocytes – $F_{1,38}$=0.10; p=0.7518, monocytes – $F_{1,38}$=0.6; p=0.1591).

The correlation coefficient of mean concentration of interleukins and the particular cells of white blood cell system

Figure 7. **Lymphocytes dynamic changes in time in study group. ES – endoscopic sphincterotomy, LCh – laparoscopic cholecystectomy**

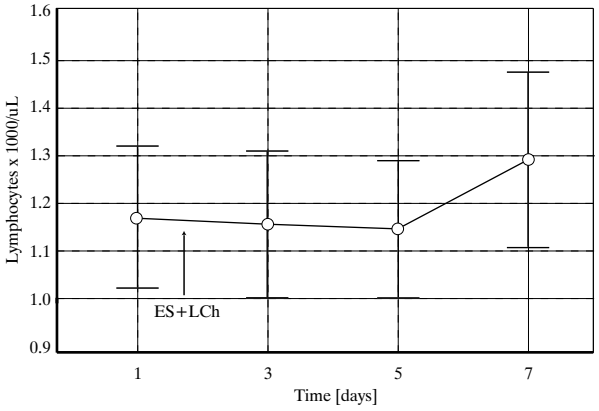
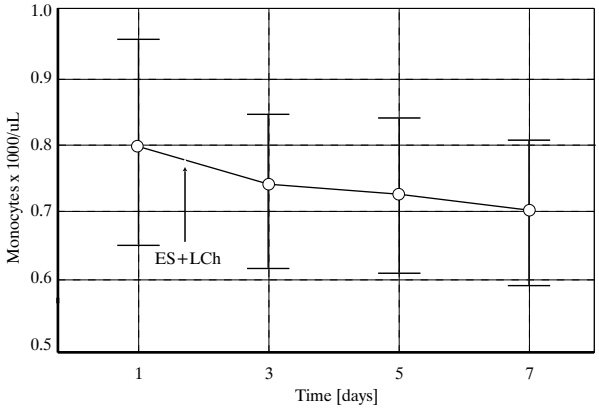


Figure 8. **Monocytes dynamic changes in time in study group. ES – endoscopic sphincterotomy, LCh – laparoscopic cholecystectomy**



in the 1st day of study was significant between IL-6 and neutrophils (correlation =0.5564; p=0.011) and between IL-6 and lymphocytes (correlation =0.4446; p=0.050). This coefficient was on the border of significance between IL-6 and the total number of leucocytes (correlation =0.4309; p=0.058). Among the particular cells of the white blood cells system the strong correlation between neutrophils, monocytes and total number of leucocytes has been shown (respectively: correlation =0.9729; correlation =0.7492 in both cases p<0.001) and between monocytes and neutrophils (correlation =0.7165; p<0.001). The rest of the correlation coefficients showed no statistical significance.

Discussion

The decrease of proinflammatory cytokines was very important, considering the therapeutic procedures which were implemented. This decrease was significantly lower for IL-6 and IL-8 after endoscopic sphincterotomy. IL-6 is a key one for inflammatory process [26]. The same behaviour was observed for IL-8, which is important for chemotactic activity of neutrophils. The positive correlation between the level of IL-6 and monocytes in the 1st day of study indirectly points to the crucial role of macrophages in the production of proinflammatory cytokines [27-29]. The observed decrease of the level of neutrophils may be connected to the implemented procedures, which definitively remove the etiological factor.

The level of TNF-α and IL-12p40 were relatively stable throughout the study. IL-12p40 probably plays a role in the development of necrotic changes in the pancreas, whereas TNF-α plays a role in the regeneration process [30]. For IL-1β we obtained only a few results which cannot be verified by statistical analysis. This is consistent with the observations of other investigators, and can be explained by the fact that this interleukin is produced by the involved organ, its half-life is very short and the tests used for its measurement are not very sensitive.

We believe that in the mild forms of acute biliary pancreatitis the improvement of bile flow is important. There are no clinical measurements which can guarantee that the stones which partially obstruct the biliary duct would not block its flow later. We observed an increase of bilirubin levels in 37 patients. We do not agree that increase of its level above 90 μmol/L is an indication for endoscopic sphincterotomy only [7]. In our patients, the lower levels were also associated with the presence of stones in the bile ducts, and its sphincterotomy produced normalisation. Many authorities in pancreatology reserve the early endoscopic sphincterotomy only for severe cases of biliary pancreatitis [7,11]. But the fact that this disease has an unpredictable clinical course makes such practice controversial. The initial clinical evaluation of the disease severity can change during the next few days. It is also known that even short but recurrent blockade of bile flow constitutes a fundamental risk factor for a severe course of pancreatitis [7]. Stone impaction may lead to severe pancreatitis or death [31,32]. The severe forms of acute biliary pancreatitis treated in our clinic will be presented in the next paper.

The small stones called microlithiasis are most dangerous. They cannot be visualized by any of the radiological methods. In addition, the oedema caused by passage of stones through the papilla of Vater can block bile flow [7]. From our experience this procedure is safe. We did not observe any complications connected with it. Moreover, the level of proinflammatory cytokines was significantly reduced after endoscopic sphincterotomy.

The following laparoscopic cholecystectomy did not influence the levels of measured interleukin, which gradually decreased. This procedure, except the removal of the reservoir of stones, allowed the objective evaluation of the pancreatitis severity. This management allowed also for a decrease of the interleukins.

This early surgical intervention enabled us to perform a laparoscopic procedure without the necessity of conversion to classical open cholecystectomy. This is in contrast to other investigators who perform cholecystectomy in the 5-7 day of hospitalization and report more than 10% conversions [21]. Also in our opinion early abdominal computed tomography in mild cases of patients with acute pancreatitis is unnecessary.

In our opinion in every patients with biliary acute pancreatitis, the endoscopic sphincterotomy should be done as early as possible and laparoscopic cholecystectomy should be performed early, because it allows the evaluation of morfological changes within the pancreas and around. The decrease of proinflammatory interleukin levels proves a benefit of the early implementation of minimally invasive techniques in management of these patients.

Acknowledgments

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Early endoscopic sphincterotomy and early laparoscopic cholecystectomy in the treatment of severe acute biliary pancreatitis – a preliminary report

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Abstract

Purpose: The proper timing of endoscopic sphincterotomy and laparoscopic cholecystectomy in acute biliary pancreatitis is still a subject of controversies.

The following rapid report presents preliminary data concerning treatment of patients with severe form of necrotizing biliary pancreatitis (SNBP) with the sequence of minimal invasive procedures (endoscopic sphincterotomy and laparoscopic cholecystectomy) performed in the first 48 hours after admission.

Material and methods: Twelve patients with SNBP were included in the study. The described above procedures were performed in all of the patients within 48 hours. We evaluated clinical outcome, complications, time of stay in hospital and also some morphological (white blood cells) and liver parameters (AST, ALT, bilirubin, ALP and GGT) of these patients in the course of the disease.

Results: Two patients died. Two other ones has local complications. We did not observe major complications after ERCP with ES and after laparoscopic cholecystectomy. Additionally, the lavage of the abdominal cavity was performed and drainage was established during laparoscopic cholecystectomy. Conversion in our group occurred in 1 person. Later complications in the course of the disease were caused by the its progression and not related to the performed procedures

Conclusions: The results are very incurable, however, performing these types of procedures in the experienced centers deserves to be taken into account.

Key words: necrotizing pancreatitis, surgical procedures, minimally invasive, endoscopy treatment.

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Introduction

The introduction of the endoscopic techniques to clinical practice created new ways of the management of patients with severe biliary pancreatitis. Early endoscopic sphincterotomy (ES) is reserved for the severe form of acute pancreatitis, especially with concomitant cholangitis [1]. Usually, the cholecystectomy is performed during the same hospital stay. In the most clinical centers it is performed during first days after the admission [2-4]. In the 2nd Chair of General Surgery of the Jagiellonian University endoscopic sphincterotomy is performed during the first 24 hours after an admission and laparoscopic cholecystectomy is performed on a next day (within 48 hours). These techniques were used in 12 patients with severe necrotizing biliary pancreatitis (SNBP). In this preliminary report we present the early results of such model of treatment.

Material and methods

Twelve patients (*Tab. 1*) with SNBP were included to the study. Mean age of the patients was 61 years (SD=17.86). All patients with SNBP underwent CT scanning. In a few cases, the CT scan was performed more than once. For each patient, the diagnosis of acute pancreatitis (AP) was established on the clinical history, ultrasound examination, and serum α-amylase activity (at least three times above the upper reference limit). All the patients enrolled to the study were hospitalized within 24 hours of the beginning of clinical symptoms. The biliary etiology of AP was determined based on the clinical history and the presence of stones in the gallbladder or in the common bile duct. The progression of morphological changes within the pancreas was evaluated using ultrasonography and the Becker scale [5]. Each patient has performed ultrasound examination every day during the study period. It allowed for the analysis of the evolution of the inflammatory changes within the gland and the surrounding areas.

The CT scans showed an evolution of necrotic changes of

Table 1. **Characterization of examined group of patients with severe necrotizing biliary pancreatitis (SNBP)**

	SNBP
Number of patients	12
Age mean (range) [years]	61 (47-86)
Sex male/female [n]	4/8
APACHE II mean	14.33
RANSON mean	4.21
CT grade (Balthazar score: A – E)	C – 3 D – 7 E – 2

Table 2. **White blood cells and liver parameters evaluated in the 1st and 7th day of the disease in the group of examined patients with severe necrotizing biliary pancreatitis (n=12)**

Measured parameter	Day 1		Day 7	
	Mean value	SD	Mean value	SD
WBC x1000/μL	13.63	2.7	13.16	7.1
AST [U/l]	175.7	219.4	60.95	24.5
ALT [U/l]	341.0	443.9	53.3	25.9
Bilirubin [μmol/l]	48.6	22.8	15.98	13.2
ALP [U/l]	443.4	303.9	306.2	129.9
GGT [U/l]	491.5	358.7	207.0	127.7

the parenchyma of the gland and in the retroperitoneal and peritoneal spaces. The Balthazar score was used for this evaluation [6]. The severity of AP was determined according to clinical and laboratory parameters. AP classification met the Atlanta criteria, Ranson's classification, and APACHE II score [7-9]. The degree of organ efficiency was measured using the MOD score (Multiple Organ Dysfunction score) [10].

Results

The mean hospitalization time was 33.8 days (SD=24.9). In all patients endoscopic sphincterotomy was performed during the first 24 hours after admission and laparoscopic cholecystectomy with insertion of drains into the peritoneal cavity for lavage was done during the next 24 hours. After introduction of such a treatment we observed in all patients the decrease of bilirubin level and the activity of liver's enzymes (AST, ALT, ALP, GGT). These results are presented in *Tab. 2*.

Two patients died. One patient (86-years old women) died after 16 days of hospitalization. In this patient the APACHE II score was 15. She died because of the respiratory insufficiency. The next patient died after 70 days of hospitalization (70-years old woman). The cause of death was multiorgan failure. In two other patients the acute pancreatic fluid collections were observed in the postoperative course. In one case the fluid resolved spontaneously, in the other one the external drainage was performed. This patient was hospitalized 78 days. One patient developed the blindness of one eye.

Discussion

The presence of stones in the biliary tree and jaundice were indications for early ES in our patients. In all patients, the ultrasound examination showed a dilatation of the common bile duct. Also the bilirubin level was increased. Endoscopic retrograde cholangiopancreatography (ERCP) is the procedure of choice in the presence of stones within biliary ducts and if performed by an experienced surgeon it is safe and effective. We observed no complications related to the ERCP with ES. Moreover, in our opinion such a treatment minimize a risk of the development of complications in the later course of a disease. During cholecystectomy not only removal of the source of stones is done but also removal of the fluid from abdominal cavity which is reach in enzymes and toxic substances. During this procedure the drainage is inserted, which allows the lavage in the postoperative period. In our opinion the early laparoscopic cholecystectomy is easier technically. Delayed cholecystectomy is more difficult because of the presence of hard, solid adhesions and more severe inflammatory infiltration of the gallbladder wall. The rate of conversion in our material is about 1% whereas in the others centers where delayed cholecystectomy is performed it, reaches 10% [2,3]. The complications caused by an infection, which developed later in the course of pancreatitis, in our opinion were connected to the progression of the diseases and not attributed to ERCP. During this procedure we always gave a broad spectrum antibiotic into the biliary tree. The presented method of management, in our opinion, deserves to be taken into account. The minimal invasive techniques develop dynamically and are widely used in the clinical practice. It is possible, that in the future such a way of treatment of patients with severe form of biliary pancreatitis will be implemented into the widely accepted algorithm of the management.

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The role of adenosine A2a receptors in experimental acute pancreatitis

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Abstract

Purpose: The role of adenosine and its receptors in acute pancreatitis remains unelucidated. The aim was to evaluate the effects of the adenosine A2a receptor agonist and antagonist in the severe, taurocholate-induced experimental acute pancreatitis (EAP).

Material and methods: The experiments were performed on 80 male Wistar rats, subdivided into 4 groups: C – the control rats, I – the EAP group, IIA – EAP group treated with the A2a adenosine receptor agonist CGS 21680, IIB – EAP group treated with the A2a adenosine receptor antagonist ZM 241385. The blood for α-amylase and lipase and tissues samples for the morphological examinations and immunohistochemistry for A2a receptors were collected in 2, 6, 24 hours of the experiment.

Results: The serum α-amylase tended to decrease in the group IIA as compared to EAP untreated after 6 and 24 h. No significant effect of both treatments on serum lipase was noted. The administration of CGS 21680 resulted in favorable decrease of the inflammatory cell infiltration, hemorrhagic changes, necrosis and vacuolization of acinar cells, without an evident effect on the edema of the interstitial tissue. The administration of ZM 241385 did not affect the scores of necro-hemorrhagic changes and inflammatory infiltration, whereas it decreased the scores of vacuolization and edema. In all groups the expression of A2a receptors was similar.

Conclusions: Our findings suggest, that A2a adenosine receptors are involved in the course of sodium taurocholate EAP. It is probable that the modulation of some subgroups of adenosine receptors could alleviate the course of severe experimental AP.

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Introduction

The essence of AP is the damage to the exocrine cells of the pancreas, with severe consequences for whole organism. This process is caused by the activation of trypsinogen. The local defensive mechanisms do not protect the organ against the effects of successively activated enzymes destroying the gland. The direct factor triggering AP and further pathogenic stages of the disease remains unknown. The studies by Satoh et al. [1] show that, via A2a receptors adenosine is likely to play an important role in the progression of edema formation in edematous, caerulein-induced EAP in rats.

Adenosine is an endogenous nucleoside that participates in a multitude of biochemical and physiological processes throughout the body. Adenosine acts through specific membranous receptors called adenosine receptors-P1 that are present in the brain, heart, kidney, blood vessels, adipose tissue and platelets [2-5]. According to their molecular and biochemical properties the receptors are divided into 4 subtypes: A1, A2a, A2b, A3. The A2a receptors are involved in of many signaling reactions in the organism. The link of nucleoside with A2a receptor on the external surface of the cell membrane results in the activation of adenylcyclase and binding with proteins Gq and Gs [6]. This mechanism is the best-known way of transmitting signals through A2a receptors.

In the alimentary tract the presence of A2a receptors was found in the longitudinal muscular layer of the intestines, parietal cells of the stomach, in the liver, pancreas and spleen [5]. In the pancreas adenosine receptors modulate both endocrine and exocrine functions [5]. Iwatsuki et al. [4,7] suggest that, through A2a receptors, adenosine modulates the secretory response of the pancreatic ductular cells.

The aim of present study was to evaluate and compare the

effects of adenosine A2a receptor agonist and antagonist in sodium taurocholate-induced EAP.

Material and methods

The experiments were performed on 80 white male Wistar rats weighing 250-300 g that were maintained for 24 h without food but were allowed free access to water. The animals were subdivided into 4 experimental groups, 20 animals in each group.

Group C – the control, healthy rats used to determine biochemical norms and standard histological images.

Group I – the rats in which EAP was induced by injecting 5% sodium taurocholate solution (Sigma Chemical Co.) at a dose of 0.08 ml/100 g b.w. into the biliary-pancreatic duct according to Aho and Henckel [8].

Group II – the rats with EAP in which the following compounds were administered (intraperitoneally) 48, 24, 12 and 1 hour before and 1 hour after injection of 5% sodium taurocholate solution into the biliary-pancreatic duet.

Group IIA – CGS 21680 A2a receptor agonist (Tocris Cookson Ltd.) each at a dose of 3 mg/kg b.w.

Group IIB – ZM 241385 A2a receptor (Tocris Cookson Ltd.) antagonist at a dose of 3 mg/kg b.w.

After 2, 6 and 24 hours the animals were anaesthetized with diazepam (0.15 mg/kg b.w.) and ketamine (5 mg/kg b.w.). The blood samples were collected from the left ventricle for biochemical tests. Samples of pancreas were obtained for histopathological and immunohistochemical examinations.

Biochemical assays

The activities of α-amylase and lipase in serum were determined by standard laboratory methods.

Histological examination

For histological examinations under light microscope, the pancreatic sections were fixed in 10% buffered formalin solution, pH 7.4. The sections were embedded in paraffin and cut into 2 μm-thick slices using a microtome. The specimens were stained with hematoxylin and eosin (H+E). Histological features of pancreatitis: the necrotic lesions in the parenchyma and adipose tissue of the pancreas, inflammatory infiltrations, erythrocyte extravasations and hemorrhages, interstitial tissue edema were assessed. The scale according to Satoh et al. [1] was used to evaluate morphological lesions in the individual groups. Histological changes of the pancreas were graded blindly (range 0-4), based on the approximate percentage of acinar cells showing vacuolization and necrosis, interstitial edema, and the approximate areas showing inflammatory cell infiltration and hemorrhage: 0=absent, 1=<5%, 2=5-25%, 3=25-50%, 4=>50%.

Immunohistochemical examination

The reactions were performed in paraffin sections with goat polyclonal antibody anti-adenosine A2A R (R-18): SC 7504 (Santa Cruz Biotechnology, Inc.). The antibodies were visualized with the Cell and Tissue Staining Kit HRP-DAB anti goat (R&D Systems, No. CTS008).

Statistical analysis

The values of biochemical parameters were statistically analyzed. The results were expressed as mean ± standard error of the mean (mean ±SEM). The data were compared using an analysis of variance (ANOVA). A 5% risk of inference error was accepted; $p<0.05$ was considered statistically significant.

Results

Biochemical assays

The results of statistical analysis of the serum α-amylase and lipase activities are compiled in *Tab. 1*. The increase in enzyme activities after the administration of 5% sodium taurocholate in group I, IIA and IIB were found to be statistically significant in comparison to control group (with the exception of the serum lipase in group I). There was some decreasing trend of serum α-amylase (n.s.) after 6 and 24 hours of EAP using the adenosine A2a receptor agonist and some increasing trend (n.s.) after 24 hours of AP using the adenosine receptor antagonist in comparison to EAP untreated. However, the decrease of α-amylase activity after 24 hours of EAP treated with the agonist in comparison to EAP treated with the antagonist was statistically significant ($p<0.05$). The changes of the serum lipase activity after both treatmens in comparison to untreated EAP were non significant.

Histological changes

The typical picture of acute pancreatitis developed during in group I during 24 hours of the experiment. Histological examination revealed that the morphologic changes of the pancreatitis were characterized by the extensive necrosis of the exocrine parenchyma, intensive interstitial edema and focal inflammatory infiltrations of various intensity consisting of neutrophils and single lymphocytes. The pathologic process spread to the peripancreatic adipose tissue in the form of enzymatic necrosis (*Fig. 1*). Damaged walls of intrapancreatic ducts with necrosis of epithelium of the ducts and damaged walls of blood vessels were found, what led to extensive erythrocyte extravasations and hemorrhages to the pancreatic parenchyma. In the cells which were not necrotic, the vacuolar degeneration of the cytoplasm was seen.

In the group IIA, treated with the A2a receptor agonist – CGS 21680, the intensity and extent of inflammatory-necrotic changes of the pancreatic parenchyma, hemorrhages and vacuolization of acinar cells were markedly decreased, while substantial interstitial edema was maintained at the same level (*Fig. 2*, *Tab. 2*)

In the group IIB, A2a receptor antagonist – ZM 241385 treated rats, neither the inflammatory-necrotic changes of pancreatic parenchyma nor hemorrhagic changes were affected by the treatment. However, the interstitial edema and vacuolization of acinar cells were evidently decreased.

The summary of the effects of adenosine A2a receptor agonist and antagonist on the histopathologic findings of EAP is reported in *Tab. 2*.

***Table 1*. The effects of adenosine A2a receptor agonist and antagonist on the serum α-amylase and lipase activities in experimental acute taurocholate pancreatitis in rats (EAP)**

		Mean	SEM	Me	Δ Me	ANOVA	p
Control (C)	Serum amylase IU/L						
		569	61.7	584			
	Serum lipase IU/L						
		24.0	7.48	22.0			
Sodium Taurocholate (EAP)	Serum amylase IU/L						
	2 h	2835	588	2679	2095		
	6 h	2069	558	1962	- 717	16.2	<0.05
	24 h	2603	248	2530	568		
	Serum lipase IU/L						
	2 h	34.4	8.20	36.0	14.0		
	6 h	34.8	11.8	31.5	- 4.50	5.0	n.s.
	24 h	41.3	17.5	34.5	3.0		
EAP + agonist A2a CGS 21680	Serum amylase IU/L						
	2 h	2230	414.7	2403	1 819		
	6 h	1041	156.2	993	-1 411	13.0	<0.01
	24 h	1883*	511.4	1842	849		
	Serum lipase IU/L						
	2 h	39.1	13.6	41.5	19.5		
	6 h	46.0	12.1	44.0	3.5	6.0	<0.05
	24 h	29.4	13.4	27.0	-17.0		
EAP + antagonist A2a ZM 241385	Serum amylase IU/L						
	2 h	1979	419	1936	1 352		
	6 h	1968	655	1848	-88.5	12.0	<0.01
	24 h	3218	286	3274	1400		
	Serum lipase IU/L						
	2 h	36.0	7.29	34.0	12.0		
	6 h	34.0	12.7	32.5	-1.50	12.0	<0.01
	24 h	27.2	9.12	30.2	26.9		

n.s. – non significant; p – statistical significance of differences in comparison to control group (C); * p<0.05 (agonist vs antagonist)

Table 2*. Histological findings of the pancreas in CGS 21680, ZM 241385 – treated rats with EAP

Time	Inflammation	Necrosis	Hemorrhage	Vacuolization of acinar cells	Edema
Sodium taurocholate EAP (untreated)					
2 h	1	1	1-2	1	1
6 h	2	2-3	2-3	2-3	2-3
24 h	3-4	4	4	3-4	3-4
EAP + agonist A2a receptor (CGS 21680)					
2 h	1	0	0	0	1
6 h	0-1	1	1	1	2
24 h	0-1	1-2	2	2	3
EAP + antagonist A2a receptor (ZM 241385)					
2 h	0-1	1	1	0-1	1
6 h	2	2	2	0	1-2
24 h	3-4	4	3	1	1

*) Histological changes of the pancreas were graded (range 0-4) based on the approximate percentage of cell for degree of inflammation, necrosis, hemorrhage, vacuolization and edema as follows: 0, absent; 1 <5%; 2, 5%-25%; 3, 25%-50%; 4, >50% [1]

Figure 1. **Group I (EAP untreated). Necrosis of parenchymal cells and adipose tissue of pancreas, hemorrhage, interstitial edema and inflammatory infiltrates. Focally, the vacuolar degeneration of acinar cells is seen. H+E (x200)**

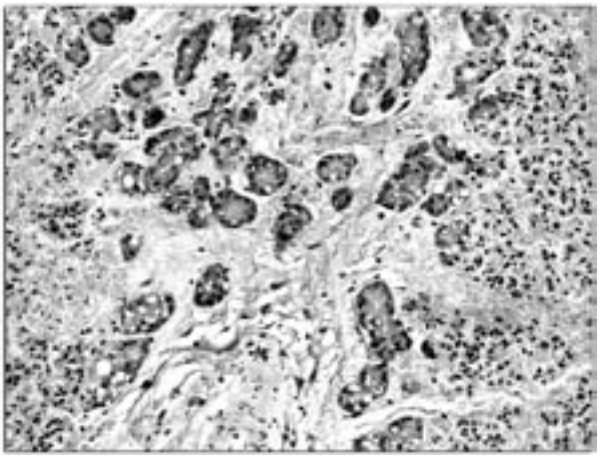
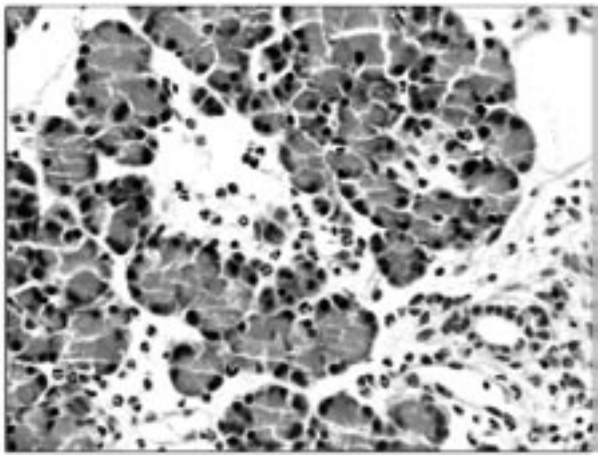


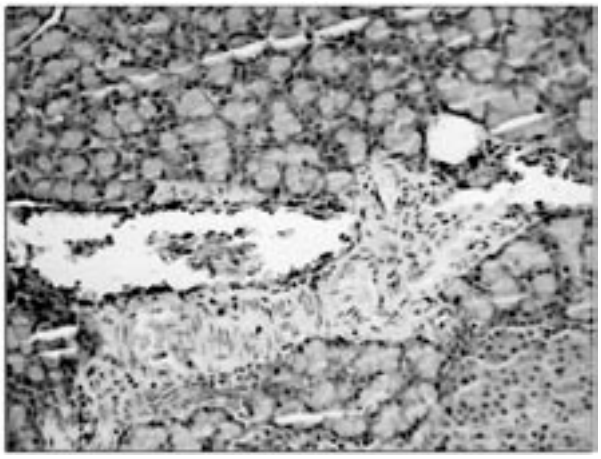
Figure 2. **Group IIA (EAP + CGS 21680). Interstitial edema and sparse inflammatory infiltrates consisting of neutrophils and lymphocytes within the parenchyma of pancreas. H+E (x400)**



Immunohistochemical examinations

The expression of A2a receptors was evaluated in the pancreas of the group C, I, IIA and IIB. In all groups the distribution of A2a receptors was similar. Their expression was observed in the exocrine part of the pancreas within the vascular endothelium (*Fig. 3*) and perivascular nerve fiber bundles. Focally, poor membranous reaction within the epithelial cells of pancreatic ducts was found. The antigen expression was also noted in the endocrine cells of islets of Langerhans located mainly on their periphery. In groups I, IIA and IIB, the expression of A2a receptors was also observed in the inflammatory cells.

Figure 3. **Immunohistochemical reaction of A2a receptors. (Group IIA). Expression of A2a receptors in endothelial cells of blood vessels (x 200)**



Discussion

The studies on etiopathogenesis and causes of unfavorable AP in some patients with necrotic-hemorrhagic form of the disease have been carried out for many years. Adenosine is a purine nucleoside, which modulates numerous physiological functions of the organism and is involved in many pathologic processes: inflammation, damage to tissue continuity, ischaemia and shock [7,9].

Recently it has been demonstrated that adenosine modulates the secretory function of the pancreas and takes part in the pathology of some pancreatic diseases, e.g. mucoviscidosis and AP [1,10]. The role of adenosine in the course of AP is poorly known. The results of the experimental studies reveal that A2a receptor-mediated adenosine may be an important modulator of the individual stages of the inflammatory process [1,11].

In this study, we investigated whether an exogenously added adenosine A2a receptor agonist and antagonist could affect the pathologic outcome of taurocholate – induced rat pancreatitis. The severity of inflammatory process was evaluated on the basis of changes in amylase and lipase activities in serum and the histological examination.

Pretreatment of EAP with the adenosine A2a receptor agonist CGS 21680, tended to decrease the serum α-amylase values after 6 and 24 hours, without statistically significant lipase changes as compared to the untreated group with EAP. In the group pretreated with the adenosine A2a receptor antagonist ZM 241385, no significant changes of α-amylase and lipase were noted.

The administration of CGS 21680 (group IIA) generally alleviated the histological scores of inflammatory processes in the pancreas. The use of highly selective A2a receptor antagonist – ZM 241385 (group IIB) did not intensify the morphological indices of this process.

In the group treated with CGS 21680, the inflammatory-necrotic changes involved only 25% of the adipose tissue and the pancreatic parenchyma after 24 h. Such picture suggests the inhibition of the inflammatory reaction in the pancreas of rats with EAP injected with the A2a receptor agonist. However, the A2a receptor agonist – CGS 21680 resulted in favorable decrease the inflammatory infiltration and necrotic lesions without such effect on the edema formation.

Similar results were reported by Satoh et al. [1] The caerulein model of mild EAP was used in their experiment. The animals were administered CGS 21680 – the selective agonist and DMPX – the antagonist of the A2a receptor. The administration of CGS 21680 also substantially decreased the inflammatory cell infiltration in the pancreas. However, it significantly increased pancreatic edema and vacuolization of the acinar cells. No statistically significant changes in amylase and lipase activities were observed. The reason of such different effects of CGS 21680 still remains unclear. In the immunohistochemical reactions of the rat pancreatic specimens with sodium taurocholate induced EAP (group I), the A2a receptors were found to be distributed on various cells. They were present in the endothelial cells, ducts epithelium, perivascular nerve plexi, pancreatic islet cells (particularly on the periphery) and cells of inflammatory infiltration. Such a varied location of A2a receptors might be responsible for both favorable and unfavorable (edema intensification) effects of CGS 21680 in the course of caerulein EAP. In contrast to their results, in our study with the severe, taurocholate EAP, the same antagonist attenuated not only inflammatory cell infiltration but also necro-hemorrhagic changes and it did not intensify the edema formation or vacuolization of acinar cells. Therefore the general effect of the adenosine A2a receptor agonist in this form of EAP seems to be beneficial.

According to Pearson [12], the main sources of adenosine in the blood are the endothelial cells. Recently, the physiological importance of the endothelium was stressed as a regulator of vasoactive purines due to the system of ecto-nucleopeptidases and the system of membranous transport for adenosine [2]. Thus, the endothelium may regulate the blood concentration of adenosine depending on its amount produced in the tissues [2,12]. Substantial interstitial edema maintained throughout our experiment, is likely to be related to the action of CGS 21680 at the level of endothelial cells.

Inoue et al. [13] suggest that the immunoregulatory action of A2a receptors takes place through their effects on the activity of neutrophils. The activation of these mechanisms of adenosine action through the A2a receptor results in decreased chemotaxis, phagocytosis and the generation of reactive oxygen metabolites by neutrophils. The next stage is the adhesion of inflammatory cells to the endothelium; further monocytes and macrophages are stimulated to secrete pro-inflammatory cytokines. According to Satoh et al. [1], in the caerulein-induced EAP, neutrophil infiltration became smaller due to the CGS 21680 treatment. In our experiment, the group treated with CGS 21680 (group IIA) also showed reduced intensity of inflammatory infiltration in the pancreas compared to the group I untreated animals. The activation of A2a receptors may have, at least partially, the protective effect in the course of AP by decreasing both the intensity of inflammatory infiltration and the release of tissue damaging factors by inflammatory cells.

The pancreatic edema in the caerulein-induced EAP was more intensive after the administration of CGS 21680 and got rapidly smaller under the influence of DMPX [1]. In our rats with taurocholate EAP infused with ZM 241385, the A2a receptor antagonist, the intensification of edema was not observed, particulary 24 hours after the induction of inflammation when the edema was markedly smaller, although the inflammatory-necrotic changes persisted and involved 50-80% of the pancreatic parenchyma. The mechanism of this selective action against edema formation is not clear.

In our experiment, no expression of A2a receptors was observed in the immunohistochemical examinations of the exocrine cells of the pancreas. Therefore, it would be reasonable to assume, that the effects exerted by the agonist or antagonist of adenosine A2a receptors are mediated by such receptors localized on non-parenchymal components of exocrine components and/or in endocrine part of the gland.

Gross et al. [14] showed that adenosine modulates the blood flow of the pancreas. The disorders of the pancreatic microcirculation are one of more important causes of AP progression [14,15]. Some authors suggest that adenosine may intensify vasospasm through the A2a receptors located on the smooth muscle cells of the capillary wall [14,15]. Other studies [1,9] reveal that A2a receptor antagonists slow down the blood flow in the capillaries dilating their lumen. An early sequel of this phenomenon may be the adherence of erythrocytes to the walls of interlobular veins preceded by a marked increase in capillary perfusion.

Thus, a decreased blood flow with simultaneous damage to the vascular wall, could increase the possibility of erythrocytes extravasation and intrapancreatic hemorrhages in EAP, which were observed in our rats with taurocholate EAP. Both, agonist and antagonist of adenosine A2a receptors, used in our study, did not aggravate the hemorrhagic changes, and even some improvement of these changes after the application of agonist was seen. The role of adenosine A2a receptors in the microcirculatory aspects of severe EAP requires further examinations.

Our findings suggest that adenosine A2a receptors are involved in the course of sodium taurocholate-induced EAP. More than one subtype of adenosine A2a receptors may be expressed on the surface of various cells. Thus blockage and stimulation of one type of A2a receptors in our experiment did not provide any clear answer to the question about the role of adenosine receptors in EAP. It is probable that the simultaneous modulation of action of several subgroups of adenosine receptors could slow down the progression of EAP, yet this requires further experimental studies.

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Metabolic effects associated with adipose tissue distribution

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Abstract

Cardiovascular and metabolic risk depends not only on the overall obesity but also fat distribution is more powerfull predictor for risk factors. Adipose tissue produces and secretes a variety of bioactive peptides – adipokines

The most recently described adipocyte secretory proteins contribute to the pathogenesis of impaired insulin secretion and insulin resistance, endothelial dysfunction, a proinflammatory state and promote progression of atherosclerosis. This review presents an overview of the adipose tissue secreted proteins (leptin, TNF-α, IL-6, adiponectin, resistin, visfatin, ASP, FIAF, MT) role and their regulation in the context of abdominal obesity and the adverse metabolic consequences.

Key words: adipose tissue, abdominal obesity, adipokines.

Obesity causes a significant increase in the morbidity and mortality rate. Obese persons are predisposed to hypertension, dyslipidaemia, diabetes mellitus and coronary heart disease [1]. Cardiovascular and metabolic risk depends not only on the overall obesity but also the fat distribution is more powerfull predictor for risk factors. Waist circumference – a convenient measure of abdominal adipose tissue, is a better predictor than BMI and waist-to-hip ratio [2]. The waist circumference is measured in the horizontal plane midway in the distance of the superior iliac crest and the lower margin of the last rib. The prevalence of obesity increases and the most alarming is markedly growing prevalence of abdominal obesity [3].

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Previously, adipocytes were considered to be an inert storage depots, storing fats as triglicerides in the fed state, and releasing fuel as fatty acids and glycerol in times of fasting [4]. Intra-abdominal adiposity liberates fatty acids directly to the portal vein, and so they have direct effects on liver metabolism. It is associated with insulin resistance leading to hiperinsulinaemia and increased hepatic triglyceride synthesis.

In the state of elevated triglicerides, LDL particles become enriched in triglycerides, which are hydrolyzed to small dense LDL particles. Increased plasma triglycerides are also associated with reduced HDL levels [5].

Adipose tissue is now known to secrete a variety of bioactive peptides – adipokines [6-8]. They act at both the local (autocrine/paracrine) and systemic (endocrine) level. Direct adverse effects of intra-abdominal adiposity occur via secretion of a rang of bioactive substances. The most recently described adipocyte secretory proteins contributed to the pathogenesis of impaired insulin secretion and insulin resistance, endothelial dysfunction, contributes to a proinflammatory state and promotes progression of atherosclerosis [9]. Leptin, tumor necrosis factor (TNF-α) and interleukin-6 secreted by intra-abdominal adiposity increases insulin resistance [6].

Leptin, secreted predominatly from adipocytes, interacts with several central neuroendocrine systems, including neuropeptyde Y leading to the inhibition of food intake. Leptin induced increase in renal sympathetic activity and blood pressure are mediated by the hypothalamic melanocortin system. Circulating leptin level is positively correlated with the body mass index. Centrally, it is capable of altering food intake, body weight, energy expenditure, and neuroendocrine function, whereas it has also peripheral effects on skeletal muscle, liver, pancreas, and other tissue. Leptin affects a diverse spectrum of metabolic processes. Leptin serves as a metabolic signal of energy sufficiency. Catecholamines inhibit leptin synthesis while leptin stimulates the sympathetic nervous system, decreases insulin sensitivity, and contributes to the development of hypertension. Leptin accelerates puberty and restores normal

gonadotropin secretion and reproductive function. Important effects of leptin include the regulation of immune function, hematopoesis, angiogenesis and the bone development [10].

Tumor necrosis factor (**TNF-α**) is a cytokine mainly produced by macrophages; adipocytes are also a significant source of TNF-α. TNF-α inhibits tyrosine kinase dependent phosphorylation of the insulin receptor, resulting in defects in insulin signaling and leading to insulin resistance and impaired glucose transport [11].

TNF-α decreases activity of lipoprotein lipase and increases hormone-sesitive lipase, preventing lipid accumulation. TNF-α exerts its effect on cell function by binding to two specific cell surface type I and type II receptors. The extracellular portions of this membrane receptors are separated from them and circulate as soluble forms. TNF-α expression in the adipose tissue and serum levels are elevated in obesity. The observed decrease of the serum concentration of TNF-α and the increase in both soluble receptors after weight reduction in obese women may be a counter-regulation preventing further weight loss [12].

Interleukin-6 (**IL-6**) has also proinflammatory activity. Both adipose tissue expression and circulating levels are correlated with obesity, impaired glucose tolerance and insulin resistance. IL-6 stimulates the liver production of CRP – important marker of vascular inflammation and predictor of atherosclerosis [8].

Adiponectin is an adipocyte-derived plasma protein with the insulin sensitizing properties. In the liver, it increases fatty acids oxidation and reduces hepatic glucose output. In the muscle, adiponectin stimulates glucose use and fatty acids oxydation. Within the vascular wall it inhibits monocyte adhesion, inhibits macrophage transformation to foam cells, increases nitric oxide production in endothelial cells. Adiponectin is an unique adipokine with antidiabetic, antiinflammatory and antiatherogenic effect. Circulating adiponectin level is reduced in obesity [13] and increases after weight loss [14].

Resistin is secreted from adipocytes. It contributes to insulin resistance [15]. Increased resistin expression in abdominal adipose tissue compared with thigh fat could explain the increased risk of diabetes associated with abdominal obesity [16,17].

Visfatin is adipokine expressed at high levels in visceral fat [18]. Visfatin stimulates glucose uptake by adipocytes and muscle cells and suppresses glucose release by hepatocytes. Visfatin binds to the insulin receptor at a different site from insulin. This cytokine exerts insulin-mimetic effects by lowering plasma glucose level. Like insulin, visfatin induces fosforylation of signal transduction proteins that operate downstream of the insulin receptor. Mice on the high-fat diet had higher plasma visfatin concentrations compared to mice fed normal chow. Plasma visfatin concentration increases during the development of obesity. Visfatin levels in serum increases parallelly with visceral but not subcutaneous fat [19]. So far only in few studies visfatin expression in adipose tissue and plasma concentration in human were assessed [20].

Our results [21] have shown an increased serum concentration of visfatin in obese women compared to controls. It may be one of counter-regulatory mechanism preventing the glucose increase during development of insulin resistance accompanying the visceral obesity.

Acylation-stimulating protein (**ASP**) is produced from three precursor proteins of the alternate complement system: C3, factor B and adipsin, which are secreted by adipocytes. ASP increases lipogenesis locally in adipocytes and inhibits hormone-sensitive lipase – mediated lipolysis. ASP level is elevated in obese humans and decreases after fasting or weight loss.

Fasting-induced adipose factor (**FIAF**) concentration increases in plasma on fasting and decreases on feeding a high-fat diet. It has been speculated that FIAF operates reciprocally to leptin.

Metallothionein (**MT**), a stress-response and metal-binding protein is synthetised particularly in the liver and kidneys. Recently MT-1 and MT-2 genes expression in adipocytes was demonstrated. The expression of the MT gene can be subjected to the adrenergic activation and was stimulated by the agents which increase cAMP. The main function of MT in adipose tissue may be an antioxidant protection of fatty acids from the oxidative damage [4].

The adverse effects of intra-abdominal adiposity occur via an increased secretion of plasminogen activator inhibitor-1 (PAI-1) which increases the risk of an intravascular thrombosis. Activation of the renin-angiotensin system and increased angiotensinogen secretion is involved in the development of hypertension in visceral obesity [9].

Obesity is associated with an increased storage of lipids in non-adipose tissue like skeletal muscle, liver and pancreatic β cells. Lipid accumulation in non-adipose cells may lead to the cell dysfunction – **lipotoxicity** [22].

Steatosis hepatis (NAFLD = non-alcoholic fatty liver disease) is found in about 60% of the obese patients, whereas NASH = non-alcoholic steatohepatits in from 20% to 25% of obese and between 2% and 3% of obese have liver cirrhosis.

Exposure of pancreatic β cells to high level of fatty acids increases insulin secretion. Long time exposure to fatty acids leads to β cells dysfunction, increased synthesis of ceramide and apoptosis. Long-term effect represents the transition from insulin resistance and glucose intolerance to overt diabetes [23].

The causes of cardiac dysfunction in obesity could be related to frequently observed hypertension and increased cardiac output; additional work load, cardiac hypertrophy and accumulation of fat pads arround ventricles, which increase ventricular stiffness and contribute to diastolic dysfunction. Cardiac intramyocytic lipid accumulation may trigger apoptosis and systolic dysfunction [24].

Obesity is associated with endothelial dysfunction. Endothelial dependent vasodilatation is impaired in proportion to insulin resistance. Visceral obesity is characterized by impaired NO-dependent relaxation in the large arteries and also loss of NO-independent, potassium-mediated relaxation in the small

Table 1. **Metabolic phenotypes**

	Amount of visceral fat	Insulin sensitivity	Plasma triglycerides
Obese with metabolic risk	high	low	high
Normal weight metabolically obese	high	low	high
Obese metabolically healthy	low	high	low
Normal weight metabolically healthy	low	high	low

arteries. Visceral obesity is a powerful predictor of coronary artery atherosclerosis [25].

Cardiovascular risk is connected with several classical risk factors, such as hypertension, hypercholesterolaemia, smoking and very important factor – abdominal obesity [26]. Adipose tissue produces and secretes inflammatory factors, which play important role in the atherosclerotic process. Abdominal obesity is often associated with other cardiovascular risk factors: hypertriglyceridaemia, low HDL-cholesterol, insulin resistance and hyperglycaemia.

The criteria of the **metabolic syndrome** from the IDF (International Diabetes Federation, 2005) requires the presence of high waist circumference, alone and two other cardiovascular risk factors among following:

- triglycerides >150 mg/dL (1.7 mmol/L)
- HDL cholesterol men <40 mg/dL (1.0 mmol/L)
  women <50 mg/dL (1.3 mmol/L)
- blood pressure >130/85 mmHg
- glucose >100 mg/dL (5.6 mmol/L) or diabetes

The ethnic-group specific values for waist circumference are provided

- for Europid men >94 cm and women >80 cm.

The risk factors recognised as criteria for the diagnosis of the metabolic syndrome [27] and novel risk factors such as chronic low grade inflammation and disturbances in the secretion of bioactive substances from adipocytes – adipokines contribute to the progression of atherosclerotic cardiometabolic disease. A triad of "new" atherogenic metabolic risk markers – fasting hiperinsulinaemia, increased apolipoprotein B concentration, and an increased proportion of small, dense low density lipoprotein particles observed in viscerally obese pattients with insulin resistance is associated with a marked increase in the risk of coronary heart disease. Simple screening variables, such as waist circumference and fasting triglyceride concentrations identify high risk, viscerally obese patients who could be carriers of the atherogenic triad [28].

Abdominal obesity increases the risk of the developing of type 2 diabetes and metabolic syndrome [29,30]. Subjects with the metabolic syndrome were between 2 and 3 times more likely to die from an adverse cardiovascular events and three times likely to have heart attack or stroke compared to people without the metabolic syndrome [31].

According to the INERHEART Study abdominal obesity is a major cause of acute myocardial infarction [32]. In the Heart Outcomes Protection Evaluation (HOPE) Study the risk of cardiovascular death, myocardial infarction, or death from any cause increased proportionally with increasing tertiles of waist circumference [33].

During 8 years of follow-up, the prospective Nurses Health Study shows relationship between the waist circumference and risk of cardiovascular disease [34].

Body fat distribution abnormalities may play an important role in the development of metabolic complications. The amount of visceral fat is associated with a decrease of insulin sensitivity, which could lead to an increase risk of cardiovascular disease.

Metabolic phenotypes [35] were identified due to amount of visceral fat, insulin sensitivity, plasma triglycerides (*Tab. 1*). Two subtypes of individuals were identified:

1) "metabolically obese" among obese and also normal weight persons,

2) "metabolically healthy" among normal weight persons and obese with normal insulin sensitivity.

Identifying the physiological and behavioral factors that could be used to classify an individual as "metabolically obese" or "metabolically healthy" would be valuable and could have important therapeutic implications.

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Magnification chromoendoscopy in comparison to standard chromoendoscopy for detection of intestinal metaplasia in renal transplant recipients

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Abstract

Purpose: Renal transplantation is associated with frequent gastrointestinal complications. Intestinal metaplasia is a feature of atrophic gastritis whereas the diagnosis of Barrett's esophagus is based on histological demonstration of specialized metaplasia. Both conditions are associated with increased risk of adenocarcinoma. The aim of the present study was to assess whether magnification endoscopy improves the diagnostic accuracy of intestinal metaplasia in stomach and in esophagus.

Material and methods: In this non-randomized, feasibility study thirty one (12 women and 19 men) renal transplant recipients, with a mean age of 44.0 years were evaluated for the presence of intestinal metaplasia. Standard esophagogastroscopy with methylene blue staining was followed by magnification endoscopy. The presence of gastritis and intestinal metaplasia was classified according to modified updated Sydney classification.

Results: Of 31 patients, 16 patients had endoscopic and histopathological evidence of gastric intestinal metaplasia, and standard endoscopy with methylene blue staining was sufficient for diagnosis (15 from 16). Magnification endoscopy allowed identification of 6 patients with specialized intestinal metaplasia in Barrett's esophagus, which would be otherwise missed.

Conclusions: In this study diagnostic accuracy of standard endoscopy for identification of intestinal metaplasia in the stomach was not improved by the use of magnification endoscopy, but the latter was an accurate method of predicting specialized intestinal metaplasia in Barrett's esophagus. The use of magnification endoscopy in the clinical setting of renal transplantation needs further studies.

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Introduction

Although the worldwide incidence of gastric cancer has declined rapidly over the recent few decades [1] it is still the second leading cause of death from cancer worldwide [2]. The highest incidence rates are seen in Eastern Asia, the Andean regions of South America, and Central and Eastern Europe, wheras its incidence in USA is one of the lowest in the world [3].

In spite of these facts surprisingly little attention is paid to this form of cancer in the transplant recipients, and no specific guidelines for screening are given. This attitude is based on the premise of only modestly increased stomach and esophagus cancer rates in the USA [4]. However, there are some data suggesting that we might need to rethink such policy. One of the most provocative findings is that in USA 5-year survival of transplant recipients (for the entire transplant recipient group) diagnosed with gastric cancer was 29% as compared with a 5% to 15% 5-year survival in the general population, finding clearly attributable to early detection [5]. Amazingly, 53% of cases were discovered incidentally during endoscopy and additional 12% during computed tomography performed for other reasons [5]. There is also small study from Asian country suggesting possibility of increased incidence of gastric cancer in renal transplant recipients [6].

Intestinal metaplasia (IM) a universal feature of atrophic gastritis is the most dependable defining morphologic feature, and is also highly relevant to the pathogenesis of atrophic gastritis [7,8]. Intestinal metaplasia is defined by the replacement of the surface foveolar and glandular epithelium in the oxyntic or antral mucosa by intestinal epithelium, which is recognized by the presence of goblet cells [7,8]. Intestinal metaplasia takes several forms, type I shows fully formed small intestinal epithelium, type II and III are incomplete, consist of goblets cells interspersed among gastric-type mucin cells. Type III of

intestinal metaplasia seems to correlate with increased risk of gastric adenocarcinoma [7]. The recent data suggest that long-term metaplasia could induce invasive carcinoma [8]. Hence reliable diagnosis of intestinal metaplasia, which is demonstrable both endoscopically and in gastric biopsy specimens may be important for early diagnosis of gastric cancer. Little is known, however, about the frequency of intestinal metaplasia in renal transplant recipients.

Despite technological advances the ability of the classical endoscopy to detect dysplastic and early cancerous changes in the upper GI tract remains limited. In conditions such as Barrett's oesophagus, practice guidelines recommend periodic endoscopic surveillance with multiple biopsies, a methodology that is hindered by random sampling error, inconsistent histopathological interpretation, and delay in diagnosis. Early diagnosis may be improved by new diagnostic modalities such as chromoendoscopy and magnification endoscopy [9].

Staining is readily available and cheap, however, adds several steps and likely several minutes to routine endoscopy. A new technique – enhanced magnification endoscopy seems to be effective, readily available method and adds only an additional 5 to 10 minutes to standard endoscopic procedures. The value of this technique is still being explored, but it seems that it may improve the detection of mucosal changes by permitting better targeting of biopsies.

This has prompted us to undertake a feasibility study in order to evaluate the value of enhanced magnification chromoendoscopy as compared with chromoendoscopy and classic endoscopy in detecting intestinal metaplasia in the stomach and in the esophagus in renal transplant recipients.

Material and methods

This was an open, non-randomized clinical study. Thirty one subjects (12 females and 19 males, aged 44.0±10.8 years), attending transplantation clinic agreed to participate in the study. Inclusion criteria were: stable graft function and 1st or 2nd renal transplantation performed more than 6 months ago (mean 2.6 years; range 0.5-20 years). The indications for upper gastrointestinal endoscopy were as follows: upper abdominal pain or discomfort (n=15), ulcer disease in the past in an asymptomatic patient (n=2) and routine endoscopy screening (performed once a year) (n=14). All endoscopic examinations were performed by a single experienced endoscopist and recorded on a videotape. At first a routine esophagogastroscopy was performed using a Fujinon GIF EG-200HR videoendoscope. The intestinal metaplasia was identified by methylene blue staining (the stain is picked up by actively absorbing tissues such as areas of intestinal metaplasia in gastric and esophageal mucosa. It does not stain nonabsorptive epithelia such as gastric mucosa). The gastric biopsies were then obtained with biopsy forceps – two from antral area, two from stomach corpus and always from the area of macroscopic changes suggesting metaplasia in stomach, or from esophagus in case of suspected intestinal metaplasia in esophagus. Mucosal surface patterns were recorded in every patient. In the next step of the examination esophagogastroscopy was repeated using Fujinon EG 485 ZW magnifying videoendoscope. The mucosa was washed of any traces of blood and additional biopsies were taken from areas suggestive of metaplasia, and the results of both examinations were compared.

Table 1. **Indications for upper gastrointestinal endoscopy in renal transplant recipients**

Indication for endoscopy	No of patients
Upper abdominal pain or discomfort	15
Ulcer disease in the past	2
Routine (performed once a year)	14

Table 2. **Intestinal metaplasia in the stomach and in esophagus identified by chromoscopy followed by routine and magnifying endoscopy**

Site of metaplasia identification	No of patients with metaplasia identified by chromoendoscopy	No of patient with metaplasia identified by chromoscopy followed by enhanced magnification endoscopy	P
Antrum	13	13	ns
Gastric corpus	2	3	ns
Esophagus	0	6	p=0.024
Total	15	22	ns

The biopsy specimens were embedded in paraffin and typical sections were obtained. The sections were stained with hematoxilin-eosin and pathologist experienced in gastrointestinal histology examined the slides. Chronic gastritis was diagnosed by the presence of mononuclear cells within the lamina propria. The presence of gastritis and intestinal metaplasia was classified according to the updated Sydney classification [10]. Rarefaction and loss of gastric glands served to assess the occurrence of mucosal atrophy. Intestinal metaplasia was defined by the presence of goblet cells and/or specialized intestinal cells [7,8].

Results

A total of 31 renal transplant recipients were examined. 16 subjects had endoscopic and histopathologic evidence of gastric mucosal intestinal metaplasia (51.6%) in antrum or in stomach corpus (13 and 3 respectively). Seven subjects had no endoscopic and histological evidence of intestinal metaplasia (*Tab. 2*).

In 15 subjects (48.4%) endoscopic evidence was consistent with histological evaluation, and diagnosis was done by standard endoscopic examination after methylene blue staining. Diagnostic accuracy of intestinal metaplasia was not significantly improved by the use of magnification endoscopy. In this group we have found only one more focus of intestinal metaplasia in the corpus of the stomach with magnification endoscopy followed by chromoscopy (*Tab. 2*). Enhanced magnification endoscopy, however, allowed identifying all 6 patients (19.4%) with specialized intestinal metaplasia in esophagus (*Tab. 2*; p=0.024 vs standard endoscopy by Fisher's exact test). All these patients had low grade dysplasia in this area identified by enhanced

Figure 1. **Endoscopic images of gastric antrum in a kidney transplant recipients without staining (left panel) and after staining (right panel) with methylene blue. Area of heterogenous staining corresponding to metaplasia (confirmed by biopsy) is seen**

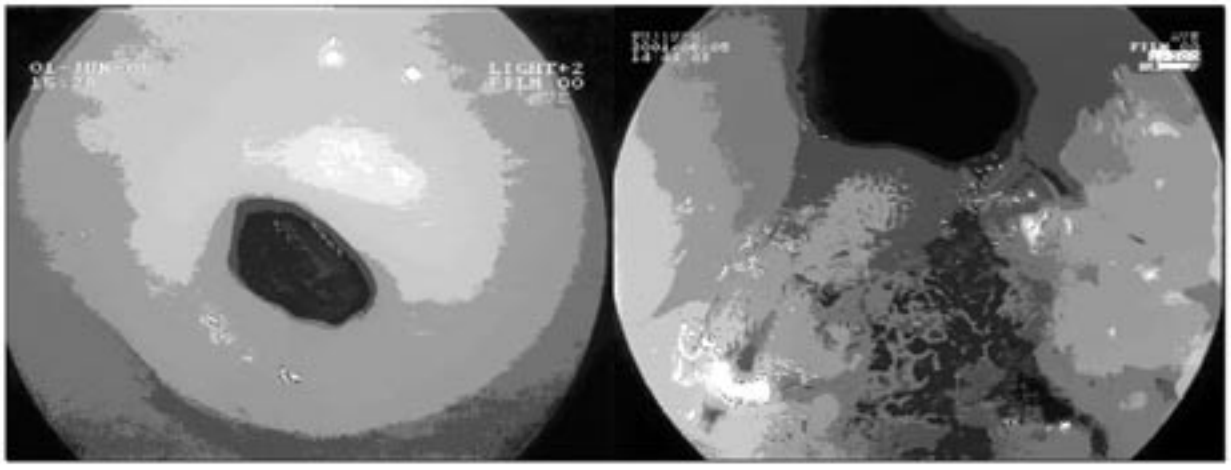
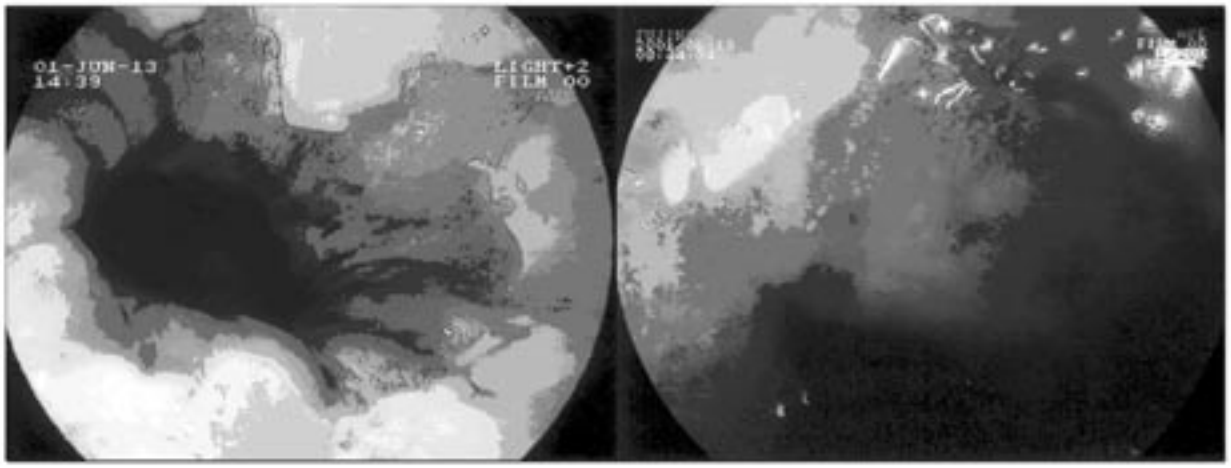


Figure 2. **Endoscopic image of specialized intestinal metaplasia in Barrett's esophagus in kidney transplant reciepients. Left panel – methylene blue chromoendoscopy. Right panel – magnification view with a tubular pit pattern**



magnification endoscopy, what has allowed diagnosing Barrett's esophagus.

Discussion

In the transplant recipient population, little is known about the clinical staging and outcome of gastric cancer and precancerous stages. The results of the present study show that in this population gastric intestinal metaplasia is a frequent finding. Its incidence in our study was similar to that reported for asymptomatic uremic patients under maintenance hemodialysis prior to kidney transplantation [11].

In this study magnification chromoendoscopy has not improved diagnostic accuracy for detection of gastric intestinal metaplasia. Standard endoscopy enhanced by methylene blue staining was sufficient. Magnification chromoendoscopy, however, significantly increased the chance of identification of specialized intestinal metaplasia in Barrett's esophagus. In fact in all cases diagnosis would be otherwise missed. This seems to have important clinical implications. It has to be borne in mind that Barrett's esophagus as a complication of gastroesophageal reflux disease (GERD) is an established precancerous condition which can lead to adenocarcinoma in the distal esophagus [12,13]. Currently, different dyes are used in conjunction with magnifying endoscopes to characterize specific surface patterns of Barrett's epithelium [13,14]. The real value of magnifying chromoendoscopy for clinical practice has not been yet determined and currently under investigation [15]. Our data suggest that these techniques have significant potential to improve diagnostic accuracy in patients with Barrett esophagus, however, were not better that routine chromoscopy in diagnosis of intestinal metaplasia in the stomach. Our results are in agreement with other investigators observations that magnification endoscopy is an accurate method predicting specialized intestinal metaplasia in Barrett's esophagus [15,16]. According to a recent paper

methylene blue staining does improve the detection of Barrett's mucosa, and areas of intestinal metaplasia are detected much more frequently than was previously recognized, even in people who were thought to have a normal esophagogastric junction on regular endoscopy [16]. According to our data the use of magnification chromoscopy is better than routine chromoscopy especially in the esophagus. This could be explained by difficulties in staining in esophagus, which is often patchy and uneven. Chromoendoscopy is a relatively new technique and depends on the skill and experience of endoscopist. Magnification in this case improves detecting of intestinal metaplasia in esophageal columnar-appearing mucosa. It seems that this technique might have impact on long-term outcome of renal transplant recipients by having a potential to improve diagnostic accuracy in patients with Barrett's esophagus, but this has yet to be proven.

In conclusion diagnostic accuracy of standard endoscopy for identification of intestinal metaplasia in the stomach is not improved by the use of magnification endoscopy, but our results suggest that this is an accurate method in renal transplant recipients for prediciting specialized intestinal metaplasia in Barrett's esophagus. Further research on the use of magnification endoscopy in renal transplant recipients in a well designed study is required to confirm our findings.

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Soluble Fas, Fas ligand and Bcl-2 in autoimmune thyroid diseases: relation to humoral immune response markers

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Abstract

Purpose: To compare soluble Fas, FasL and Bcl-2 in Graves' disease (GD) and Hashimoto's thyroiditis (HT) to the markers of humoral response: aTPO, aTG and aTSHR.

Material and methods: 5 groups of subjects: 1) 14 patients with GD in euthyreosis on methimazol (euGD); 2) 20 patients with hyperthyroid GD (hrGD); 3) 15 patients with HT in euthyreosis on levothyroxine (euHT); 4) 16 patients with hypothyroid HT (hoHT); 5) 12 healthy volunteers age and sex-matched to group 1-4. Serum concentrations of Fas, FasL, Bcl-2, aTPO and aTG were determined by ELISA and aTSHR by RIA.

Results: Levels of sFas were the highest in hoHT: 8.7 (7.2-9.8) ng/ml as compared to the controls ($p<0.01$) and euHT ($p<0.05$). We found positive correlations between sFas and aTPO in all studied groups ($r=0.25$, $p<0.05$) and between sFas and TSH in HT ($r=0.4$, $p<0.05$). In GD there was a positive correlation between sFasL and aTG ($r=0.5$, $p<0.01$) and negative correlations between sFasL and Fas ($r=-0.39$, $p<0.01$) and between sFasL and period of methimazol administration ($r=-0.32$, $p<0.05$). Levels of sBcl-2 were significantly increased in euHT: 31.0 (13.5-44.1) ng/ml as compared to the controls ($p<0.05$) and euGD ($p<0.05$).

Conclusions: Fas/FasL mediated apoptosis plays an important role in the active stage of the autoimmune process of both Hashimoto's thyroiditis and Graves' disease, however, in Hashimoto's thyroiditis they contribute to irreversible damage of thyreocytes. Early detection of Hashimoto's and Graves' diseases allows for the initiation of the proper treatment that probably leads to the reduction of the autoimmune process intensity.

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Key words: sFas, sFasL, sBcl-2, Graves' disease, Hashimoto's thyroiditis.

Introduction

Autoimmune thyroid diseases (AITD) constitute a group of the most common illnesses caused by autoaggression of the immune system, comprising up to 1.5% of the human population [1]. Pathogenesis of Hashimoto's thyroiditis (HT) and Graves' disease (GD) remains unclear in spite of considerable number of studies. Recently published papers support importance of apoptosis mediated by the interaction between Fas ligand (FasL) and Fas present on the follicular cells surface in cytotoxity, leading to thyrocytes destruction in Hashimoto's thyroiditis [2,3]. There is also some data for thyroid-cell apoptosis in Graves' disease [4,5]. Moreover, evidence from animal models of AITD suggests a role of FasL/Fas dependent cell death in selection of immunocompetent cells in the pathogenic process of HT and GD [6]. Fas (CD95, APO-1) as well as FasL (CD95L, CD178) are members of the Tumor Necrosis Factor (TNF) family and are expressed both on activated T lymphocytes and on thyrocytes of patients with AITD [7]. In autoimmune process combination of inflammatory cytokines: TNFα, interferon γ and interleukin 1β may activate expression of FasL on T cells and may sensitized thyroid follicular cells [8]. FasL/Fas pathway may be regulated at different levels including the expression of the Fas receptor and its cleavage, the inhibition of intracellular death domains and the modulation of Bcl-2, an important anti-apoptotic agent [9]. Role of Bcl-2 in AITD is still being studied and needs to be clarified [10]. Markers of humoral immune response: antithyroperoxidase antibodies (aTPO), antithyroglobulin antibodies (aTG) and antithyroropin receptor antibodies (aTSHR) take important part in clinical assessment of patients with HT and GD as a consequence of well known role of their role in pathogenesis of AITD.

Thus, the aim of the study was to compare soluble Fas, FasL and Bcl-2 in GD and HT in relation to aTPO, aTG and aTSHR

Figure 1. **The median and interquartile ranges of the serum Fas levels in patients with euthyroid Graves' disease (n=14), hyperthyroid Graves' disease (n=20), euthyroid Hashimoto's thyroiditis (n=15), hypothyroid Hashimoto's thyroiditis (n=16) and healthy controls (n=12)**

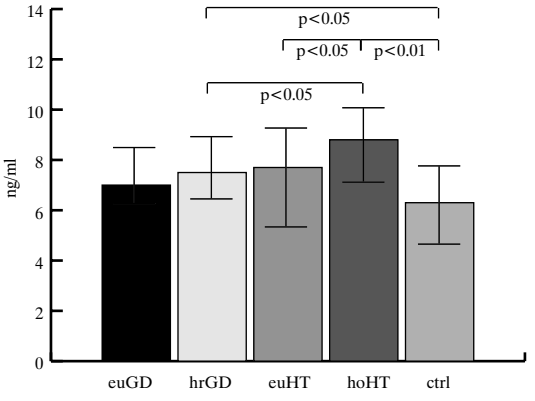
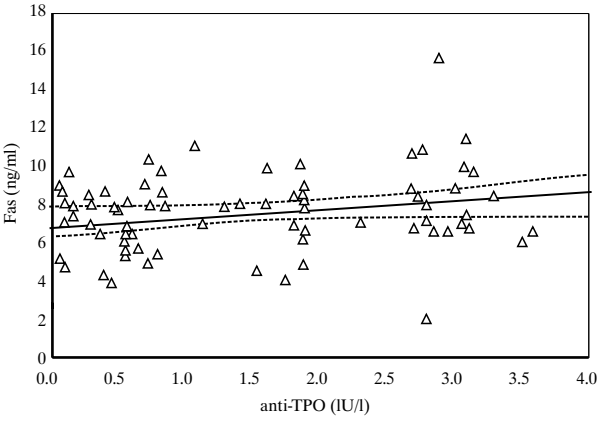


Figure 2. **Positive correlation between serum Fas and aTPO in all the studied individuals (r=0.25, p<0.05)**



to assess a role of proapoptotic Fas/FasL system and antiapoptotic protein Bcl-2 in pathogenesis of AITD.

Material and methods

The study was carried out in 5 groups of subjects: 1) 14 patients with GD in euthyreosis treated with methimazol (euGD): 11 females and 3 males in mean age 41±15 years with a duration of the disease from 7-25 months; 2) 20 patients with hyperthyroid GD (hrGD): 12 females and 8 males aged 42±23 years with a duration of the disease from 3-12 months; 3) 15 patients with HT in euthyreosis treated with levothyroxine (euHT): 15 females in mean age 45±21 years with a duration of the disease from 12-134 months; 4) 16 patients with hypothyroid Ht (hoHT): 14 females and 2 males in mean aged 53±22 years with an diagnosis of the disease 1-12 months prior to the study: and 5) 12 healthy volunteers age and sex-matched to group 1-4 (ctrl): 8 females and 4 males aged 42±18 years who had either no family history of Graves' disease nor other autoimmune diseases. Clinical euthyreosis in groups of 1 and 3 was confirmed by thyrotropin and free thyroxine estimation. Among 77 studied individuals 60 of them (78%) were women to reflect the prevalence relation of AITD in females to males that accounts for 4 -10:1. No acute infections were observed in the studied subjects 3 weeks prior to the study.

All the sera were kept frozen at -70°C until used. The serum levels of Fas, FasL, Bcl-2, aTPO and aTG were determined by the ELISA commercial kits: Fas (Quantikine kit, R&D, Mineapolis, USA; sensitivity 20 pg/ml; intra-assay precision (CV) 3.8%) and FasL (Quantikine kit, R&D, Mineapolis, USA; sensitivity 2.66 pg/ml; CV 4.7%), Bcl-2 (Bender Medsystems, Viena, Austria; sensitivity 0.5 ng/ml; CV 8.6%), aTPO and aTG (DIAMED, Warsaw, Poland; sensitivity respectively 40 IU/ml and 60 IU/ml). The serum levels of aTSHR were determined by the RIA method (TRAK kit, BRAHMS, Berlin, Germany; sensitivity 0.9 IU/L; CV 7.0%). The statistical significance was estimated by Mann-Whitney U-test and to evaluate relationships between variables Spearman's test was performed using Statistica 6.0 for Windows XP (StaSoft, Tulsa, USA).

Results

The results of serum Fas, FasL and Bcl-2 levels in the examined groups are shown in ng/ml as medians and interquartile ranges. Levels of sFas were higher in all the studied groups as compared to the controls, however, the highest values were found in hoHT individuals: 8.7 (7.2-9.8) as compared to the controls: 6.6 (4.4-8.0) ($p<0.01$) and euHT patients: 7.7 (5.2-8.7) ($p<0.05$). There was also a significant difference between hrGD group: 7.6 (6.6-8.5) in comparison with the controls ($p<0.05$) and hoHT patients ($p<0.05$). Relations in sFas values between studied groups are shown in *Fig. 1*.

We found a significant positive correlation between sFas and aTPO in all studied patients ($r=0.25$, $p<0.05$) (*Fig. 2*). There was also a positive correlation between sFas and TSH in HT patients ($r=0.4$, $p<0.05$) (not shown).

There were no significant differences in sFasL concentrations between studied groups: euGD 9.5 (8.5-11.3), hrGD 10.1 (8.4-11.4), euHT 9.7 (8.4-10.3), hoHT 10.7 (7.0-11.3) and the controls 9.7 (8.8-10.9). However, in GD patients we found a positive correlation between sFasL and aTG ($r=0.5$, $p<0.01$) (*Fig. 3*) and negative correlations between sFasL and Fas ($r=-0.39$, $p<0.01$) (*Fig. 4*) and between sFasL and period of methimazol administration ($r=-0.32$, $p<0.05$) (not shown).

Levels of sBcl-2 values were increased in all the AITD groups however significantly higher sBcl-2 values were found only in euHT: 31.0 (13.5-44.1) as compared to the controls: 8.0 (5.0-18.9) ($p<0.05$) and euGD patients: 9.1 (6.6-19.0) ($p<0.05$) (*Fig. 5*).

We found a negative correlation between sBcl-2 and aTPO ($r=-0.38$, $p<0.05$) (*Fig. 6*).

We found no correlation between soluble Fas, FasL and Bcl-2 and thyrotropin and free thyroxine concentration and goiter size.

***Figure 3*. Positive correlation between serum FasL and aTG in GD patients (r=0.52, p<0.01)**

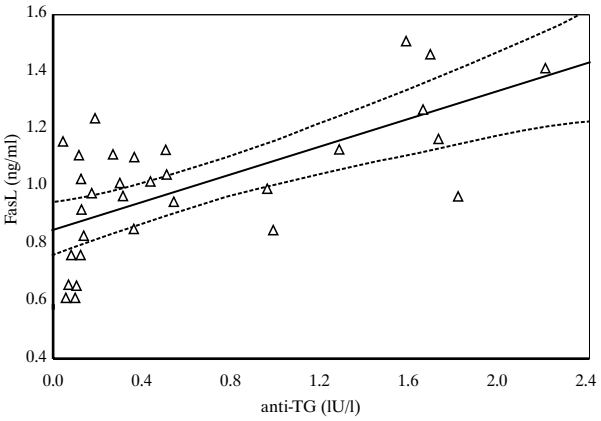


***Figure 4*. Negative correlation between serum FasL and Fas in GD patients (r=-0.39, p<0.01)**

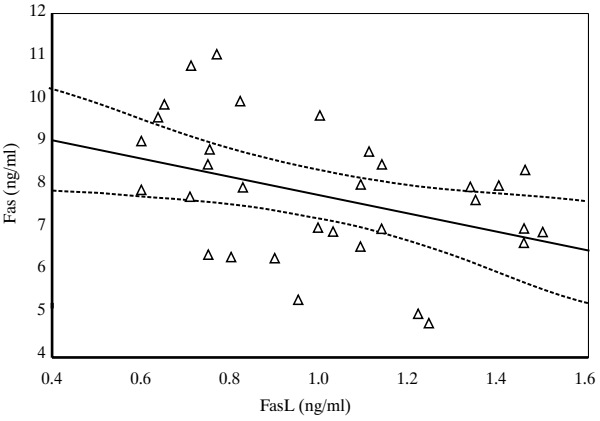


***Figure 5*. The median and interquartile ranges of the serum Bcl-2 levels in patients with euthyroid Graves' disease (n=14), hyperthyroid Graves' disease (n=20), euthyroid Hashimoto's thyroiditis (n=15), hypothyroid Hashimoto's thyroiditis (n=16) and healthy controls (n=12)**

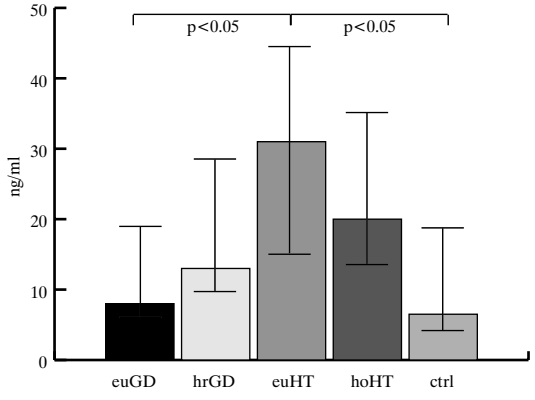
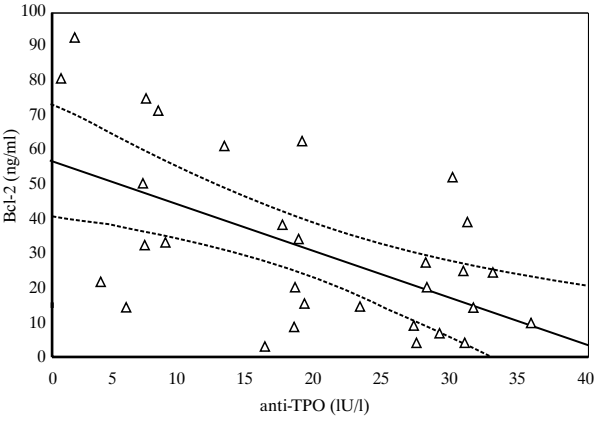


***Figure 6*. Correlation between serum Bcl-2 and aTPO in HT patients (r=-0.38, p<0.05)**



Discussion

Our data have shown increased sFas levels in all the AITD patients, however, the highest concentrations were found in hoHT group. Fas expressed in cell membranes and predisposing to FasL mediated apoptosis may be present both on thyrocytes of patients with AITD and infiltrating lymphocytes. Thus, Fas/ /FasL interaction considerably influences destruction of thyroid tissue on one side and takes important part in the elimination of autoreactive lymphocytes on the other side [10]. Circulating forms of Fas origin both due to the proteolytic cleavage from transmembrane domains and direct mRNA transcription [11]. The results of the recently published studies on the role of soluble Fas and FasL in pathogenesis of AITD remain unequivocal [12,13]. Circulating Fas is commonly considered as a factor that inhibits membrane Fas mediated apoptosis [11]. Our data showing an increase in sFas concentration especially in patients with hypothyroid HT may be a consequence of the enhanced releasing of circulating forms of Fas that seem to reflect the intensity of Fas/FasL mediated apoptosis. As shown by Salmaso et al. in HT apoptosis in a higher degree involves thyrocytes than infiltrating lymphocytes that is connected with a more intense Fas expression on follicular cells [10]. We have found in HT patients positive correlations between TSH and sFas as well as between TSH and aTPO. These findings suggest a relationship between the autoimmune process activity and a degree of hypothyreosis. Moreover, taking into account cytotoxic capacity of aTPO, positve correlation between sFas and aTPO in HT patients suggest that increased sFas may reflect an intensity of the immune involved destruction of the thyroid follicular cells.

Our data on elevated sFas in GD patients is in line with results of Hara et al. which show higher values of circulating Fas in hyperthyroid patients and a positive correlation between sFas and free thyroxine as well as aTSHR [14]. Hiromatsu et al. found a decrease in sFas level in hyperthyroid GD treated with antithyroid drug and a correlation only between sFas and aTSHR, but no correlation between soluble Fas and free triiodothyronine, free thyroxine, thyroid iodine uptake, TSH and aTPO [11]. Antibodies aTSHR are considered an important antiapoptotic factor that may protect thyreocytes of patients with GD from Fas/FasL mediated apoptosis [15]. However,

we did not find any correlation between sFas and aTSHR or thyreometabolic parameters. Our results are in line with data of Takeda et al. [12]. Feldkamp et al. suggest that elevated sFas concentration is a consequence of hyperthyreosis independently on the causes of thyreotoxicosis [16].

Circulating FasL is considered to maintain the capacity to stimulate apoptosis [17]. Taking into account the increased "antiapototic" sFas level in active GD patients, a negative correlation between sFasL and sFas seem to be compliant with findings of a few apoptotic thyrocytes in patients with GD [11]. We have found also a positive correlation between FasL and aTG concentration in GD patints. This data may suggest a role of Fas/FasL dependent apoptosis in autoreactive lymphocyte B selection. Moreover, there was a negative correlation between sFasL and a period of methimazol administration in GD. Antithyroid drugs were shown to manifest immunomodulatory actions. Mitsiades et al. demonstrated the enhanced expression of FasL on thyrocytes in GD patients treated with methimazol [18]. These findings suggest that antithyroid drugs may "arm" thyroid follicular cells with FasL to overcome immunocompetent cells and in consequence to extinguish autoimmune process with accompanied Fas/FasL mediated apoptosis.

Bcl-2 suprafamily proteins were documented to play a crucial role in regulation of apoptosis [18]. Antiapoptotic Bcl-2 expression was shown to be increased in thyrocytes of GD patients in comparison to HT patients and inverse relationship between expression of Bcl-2 on membranes of infiltrating B lymphocytes in GD and HT [11,19]. Moreover, Mitsiades et al. have found thyrocytes in HT to express Bcl-2 less intensively than normal thyroid follicular cells [20]. In spite of numerous studies on membrane Bcl-2 in AITD, data on its circulating form is lacking. We found elevated level of sBcl-2 in HT patients in which euthyreoid state was maintained by levothyroxine. Moreover there was a negative correlation between sBcl-2 and aTPO in HT, suggesting inhibitory influence of Bcl-2 on humoral immune response and thyrocyte destruction, as far as aTPO demonstrates cytotoxic capacity [9]. It may be also speculated that increased sBcl-2 accompanied with decreased sFas level in HT patients in euthyroid state due to levothyroxine may be connected with "protective" influence of exogenes thyroid hormone administration in HT. Slowering of thyrocyte metabolism caused by exogenes levothyroxine may decrease its antigenicity and autoimmune cytotoxic response as it was shown for low-dose insulin in first-degree relatives of type 1 diabetes patients in protection of pancreatic β cells [21].

In summary our results suggest that mechanisms of apoptosis mediated by interaction of Fas and its ligand play an important role in the active stage of the autoimmune process both in pathogenesis of Hashimoto's thyroiditis and Graves' disease, however in Hashimoto's disease it seems to cause irreversible damage of thyreocytes. Early detection of both Hashimoto's thyroiditis and Graves' enables initiation of the proper treatment that probably contributes to the reduction of the autoimmune process intensity.

Acknowledgments

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Does smoking affect thrombocytopoiesis and platelet activation in women and men?

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Abstract

Purpose: Smoking is a significant risk factor of cardiac ischaemia. Changes in platelet count, morphology and platelet activation enhance the risk.

Material and methods: The objective of the study was to assess platelet parameters in smoking healthy subjects with reference to sex. In the group of women, 27% were smokers, in the group of men – 49%. All the subjects were tested for platelet count (PLT), mean platelet volume (MPV), percentage of large platelets (L_{PLT}), concentrations of β-thromboglobulin, sP-selectin (soluble) and thrombopoietin, percentage of reticulated platelets (RP) and absolute count of reticulated platelet.

Results: Lower platelet count (237.00±39.52 vs 258.34±40.81 x 10^9/l, p=0.0002), higher percentage of reticulated platelets (1.39±0.66 vs 1.04±0.35%, p=0.04) and higher concentration of sP-selectin (52.66±18.54 vs 43.94±17.14 ng/ml, p=0.03) were observed only in the group of smoking women, compared to non-smokers. In neither of the sexes smoking had an effect on the following parameters: mean platelet volume, percentage of large platelets, concentration of thrombopoietin, absolute count of reticulated platelet and concentration of β -thromboglobulin.

Conclusions: The results allow the hypothesis that women are more sensitive to smoking than men. Platelets in male smokers are less sensitive to smoking – the study showed no significant changes in the parameters.

Key words: platelet count, parameters of thrombocytopoiesis, platelet activation, smoking.

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Introduction

Tobacco smoking is one of the major factors accelerating atherosclerosis [1]. Deleterious effects of smoking are associated with generation of free radicals that break down NO, which on the one hand enhances thromboxan synthesis (the prothrombic action), but on the other reduces production of prostacyclin (the antithrombic action), thus leading to clotting disorders, additionally enhanced by increased production of fibrinogen and factor VII [2,3]. According to Tsiara et al., the increased risk of atherosclerosis and thrombotic disorders in chronic smokers is not only caused by the inhibition of NO release by platelets but also by enhanced production of PECAM-1, which stimulates migration of monocytes along the endothelium [4]. Moreover, smokers show increased potential for oxidation of lipids, particularly LDL cholesterol, which is then phagocytised by macrophages and finally constitutes an important component of atheroma. It has been also found that smoking leads to the inhibition of lecithin-cholesterol acyltransferases (present on the surface of HDL cholesterol molecules in combination with apolipoproteins), which contributes to a decline in the reversible transport of cholesterol, to an increase in concentration of lipoproteins rich in triglycerides (LDL) and to a decrease in HDL concentration [1]. Additionally elevated blood carboxyhaemoglobin level causes ischaemia and vascular endothelial damage. According to Bazzano et al., a strong positive correlation between smoking and elevated levels of reactive protein C, fibrinogen and homocystein indicates that inflammation and hyperhomocysteinaemia are an important mechanism through which smoking enhances atherosclerosis.

Undoubtedly, the unfavourable influence of both active and passive smoking is connected with the effect on platelets. Addicted smokers show increased potential for platelet aggregation, lower platelet survival rate and increased excretion of thromboxan metabolites [1,4]. Elevated platelet aggregation induced by passive smoking may cause an increase in the risk of cardiac ischaemic disease even by 34% [2].

Research data concerning the effect of smoking on platelet parameters, including activation, are equivocal and do not take sex into consideration. Besides, there are very few reports on the effect of smoking on thrombocytopoiesis. Given the above, we decided to assess the effect of smoking on thrombocytopoiesis, platelet activation and some morphological parameters in healthy male and female blood donors.

Material and methods

The study group consisted of 125 healthy blood donors (mean age 31.95 years), who reported to the Regional Centre of Blood Donation and Haemotherapy in Białystok. There were 60 women (mean age 30.85±10.32 years) and 65 men (mean age 33.06±9.01 years) in the group. Subjects who had been taking antiplatelet drugs for at least 10 days prior to blood collecting and those suffering from heart and/or circulatory system disorders and diabetes were excluded from the study. Referential blood morphology parameters were within the norm in all the study groups. The subjects were divided into 4 groups: F_1 – female smokers (16; mean age 32.94±11.05 years), F_2 – female non-smokers (44; mean age 29.48±10.21 years), M_1 – male smokers (32, mean age 33.94±9.15 years) and M_2 – male non-smokers (33, mean age 32.21±8.95 years).

The following platelet parameters were assessed: platelet count (PLT), mean platelet volume (MPV), percentage of large platelets (L_{PLT}), concentration of β-thromboglobulin (β-TG) and sP-selectin, concentration of thrombopoietin (TPO), percentage of reticulated platelets (RP) and absolute count of reticulated platelet and their correlation with smoking.

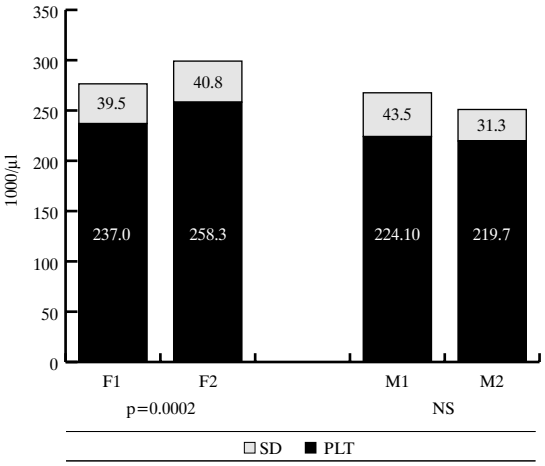
The material for analysis consisted of venous blood collected at 8-9 a.m., without stasis (to avoid platelet activation). Morphology and such platelet parameters as PLT, MPV, L_{PLT} were assessed in blood samples collected for EDTA-K_2, on haematological analyzer Advia 120 (Bayer). ELISA method was used to analyse the concentrations of β-thromboglobulin and sP-selectin (soluble) in venous blood collected to Vacutainer-type test-tubes containing anticoagulant CTAD. ELISA method was also used to determine thrombopoietin concentration in the blood collected for clot. The percentage of reticulated platelets was determined using a flow cytometer Epics XL, Coulter, with thiasole orange and CD 41Pe antibodies.

Study results were statistically analyzed based on the STATISTICA 8.0 PL. The hypothesis of normal distribution was verified with Kolomogorov test. Differences between the groups were evaluated with t-Student test for non-paired values. The level of $p<0.05$ was considered statistically significant. The correlation intensity for two variables was expressed by means of Pearson's correlation coefficient.

Results

The platelet count in the group F_2 (female non-smokers) was statistically significantly higher ($p=0.0002$) 258.34±40.81 x 10^9/l, compared to female smokers 237.00±39.52 x 10^9/l. However, no statistically significant difference was noted in platelet count between male smokers and non-smokers (*Fig. 1*). In neither of the sexes smoking had an effect on the following parameters: mean platelet volume, percentage of large platelets, thrombopoietin concentration, absolute count of reticulated platelet and β-thromboglobulin concentration (*Tab. 1* and *2*). The percentage of reticulated platelets was statistically significantly higher ($p=0.04$) in female smokers 1.39±0.66% in comparison to female non-smokers 1.04±0.35 %. In men, however, the percentage of reticulated platelets was similar in both male groups and did not show any statistically significant differences (*Fig. 2*). The concentration of sP-selectin in the group of women was statistically significantly higher ($p=0.03$) in smokers 52.66±18.54 ng/ml as compared to non-smokers 43.94±17.14 ng/ml. No statistically significant differences in this parameter were found in the group of men (59.58 ± 22.65 ng/ml in smokers and 53.62 ± 16.86 ng/ml in non-smokers) (*Fig. 3*).

Figure 1. **Platelet count in group of women (F1 – smoking, F2 – non smoking), and men (M1 – smoking, M2 – non smoking)**



Discussion

Literature reports on the effect of smoking on platelet count seem to be controversial. Brummit et al. found no correlation between platelet count and smoking in healthy volunteers [5]. Also Dotevall et al. noted no changes in platelet count in female smokers and non-smokers [6], and Suwansaksri et al. observed no alterations in PLT in male smokers and non-smokers [7]. According to Blann et al., smoking two cigarettes a day by chronic smokers of both sexes does not affect the platelet count [8]. No correlation has been also found between platelet count in pregnant women and the urinary level of nicotine metabolites [9]. However, Chao et al. have revealed that chronic male smokers have elevated PLT compared to male non-smokers [10].

In the current study, female smokers accounted for 27% of all the women involved, while in the group of men 49% smoked. We found lower platelet count, higher percentage of reticulated platelets and higher level of sP-selectin in the group of female smokers as compared to non-smokers. In the group of men,

Table 1. **Thrombopoietin concentration and absolute count of reticulated platelet in group of women (F1 – smoking, F2 – non smoking), and men (M1 – smoking, M2 – non smoking)**

	F_1 n=6	F_2 n=44	M_1 n=32	M_2 n=33
TPO (pg/ml)	145.32±36.61	161.47±64.08	177.23±63.74	182.53±58.50
absolute count of RP (x 10^9/l)	3.26±1.45	2.87±1.03	2.54±1.13	2.84±1.16

Table 2. **Mean platelet volume, large platelet count, β-thrombpoietin concentration in group of women (F1 – smoking, F2 – non smoking), and men (M1 – smoking, M2 – non smoking)**

	F_1 n=16	F_2 n=44	M_1 n=32	M_2 n=33
MPV (fl)	8.58±0.83	8.94±1.01	8.98±1.16	8.90±0.84
L_{PLT} (%)	6.42±4.12	6.88±3.95	6.84±4.32	6.93±3.58
β-TG (IU/ml)	216.68±54.34	223.59±36.82	230.98±27.49	222.59±37.28

Figure 2. **Reticulated platelet count in group of women (F1 – smoking, F2 – non smoking), and men (M1 – smoking, M2 – non smoking)**

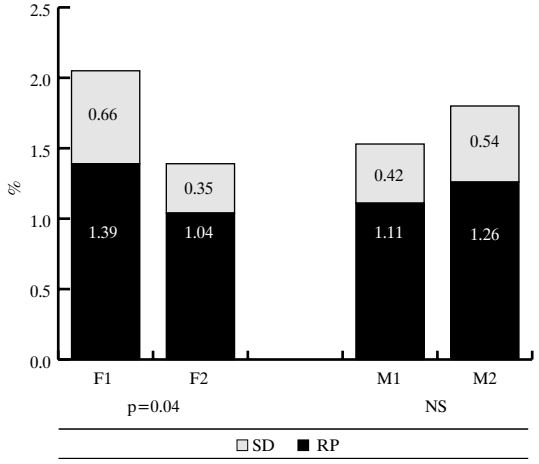
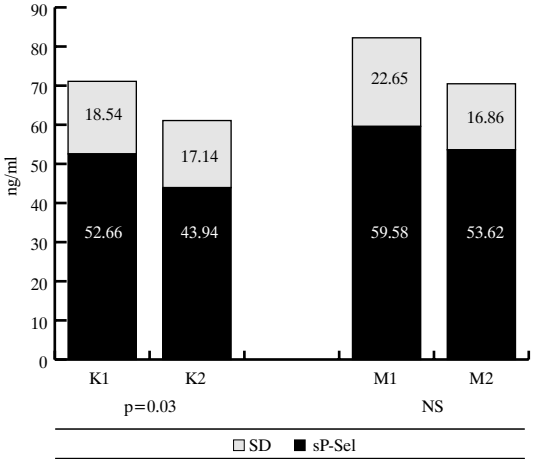


Figure 3. **Soluble P-selectin concentration in group of women (F1 – smoking, F2 – non smoking), and men (M1 – smoking, M2 – non smoking)**



platelet count was slightly higher (statistically insignificantly) and sP-selectin level higher in smokers than in non-smokers. Additionally, male smokers had minimally lower percentage of reticulated platelets, absolute count of reticulated platelet and thrombopoietin level, as compared to non-smokers. The lower platelet count in female smokers as compared to non-smokers in the current study is difficult to explain. Lack of changes in TPO concentration suggests that the observed phenomenon does not reflect thrombocytopoietic disturbances in female smokers in comparison to non-smokers. It is only the higher percentage of reticulated platelets that may indicate accelerated platelet restoration. Higher platelet turnover means the appearance of younger, more metabolically active platelets in the circulation. Compared to mature platelets, they have greater density and show higher expression of surface receptors [11]. Therefore, lower platelet count and higher percentage of young reticulated platelets in female smokers seem to be an unfavourable event, increasing the likelihood of platelet activation.

Our assumptions have been confirmed by higher sP-selectin concentrations in female smokers compared to non-smokers obtained in the current study. Similarly, Nair et al. demonstrated increased expression of P-selectin and sP-selectin concentration in smokers, which proved platelet activation [12]. Also Ridker et al. found a strong positive correlation of sP-selectin concentration with smoking [13]. However, Barbaux et al., studying a group of patients suffering from coronary diseases, noted significantly higher values of sP-selectin in smokers (135.9 ng/ml) as compared to non-smokers (123.4 ng/ml) [14]. According to this author, this is consistent with the effect smoking exerts on inflammatory markers, the adhesive molecules in particular. However, in a study by Ponthieux et al., men had higher levels of sP-selectin than women, and no effect of smoking on this marker

was found [15]. Conwey et al. demonstrated a positive correlation between sP-selectin concentration and smoking, although the level of this marker of activation was lower in women than in men [16]. Blann et al. found no changes in sP-selectin level in addictive smokers of both sexes, as compared to non-smokers [8]. Undoubtedly, smoking exerts a harmful effect on platelets, the effect being especially visible after even short-term abstinence from smoking. In healthy subjects, already after 6 weeks following smoking cessation, plasma sP-selectin concentration decreased by 29% [17]. Platelet aggregation potential improves in long-term smokers already after 2 weeks following smoking cessation [18].

We found no alterations in β-TG in the plasma of smoking women as compared to non-smokers. Similar results were obtained by Dotevall et al. [6]. At the same time, these authors observed increased urinary excretion of TXB_2 metabolites, which may indicate that these parameters are more sensitive markers of platelet activation than β-TG [6]. Smoking was also found to cause an increase in β-TG concentration in the urine and epinephrine in the blood of men with arterial hypertension as compared to the control group of subjects with normal blood pressure [19]. An increase in α granules: PF-4 and β-TG in the blood was observed in healthy smokers [4]. Chao et al. demonstrated that chronic male smokers had higher concentration of platelet factor 3 (PF-3) released from blood platelets [10]. However, Doteval et al., in a study involving a group of young healthy smoking women, found no changes in PF-4 concentration in the blood and β-TG in the blood and urine, in the mean platelet survival rate and in platelet production, as compared to non-smokers [6]. We found no effect of smoking on MPV and L_{PLT}. However, Calori et al. demonstrated that in monozygotic twins of both sexes smoking leads to increased MPV [20]. The changes observed in blood platelets are in the author's opinion due to the effect of smoking on vascular endothelium.

The data presented here allow the hypothesis that women show greater sensitivity to smoking than men. Responsible for this mechanism is probably the antiestrogenic effect of smoking on blood platelets. Smoking induces conversion in estradiol transformations, which leads, through the effect on P_{450} isoenzyme, to the production of metabolically inactive compounds [4]. According to Tsiara et al., especially in young and middle aged women, smoking enhances the risk of cardiovascular diseases, as compared to men [4]. Ultimately, irrespective of sex, the incidence of heart infarct and mortality rates due to ischaemic disease are higher in smokers than in non-smokers, especially under 65 [1].

Based on the current study and literature data, it cannot be definitely stated that nicotine and other cigarette components cause platelet activation. In smokers, platelet activation can be associated with structural or biochemical alterations in the circulation (changes in endothelium, increase in free fatty acids) rather than with direct effect of tobacco on platelets [21].

Conclusions

Women demonstrate higher sensitivity to smoking than men – differences in platelet count, parameters of thrombocytopoiesis and platelet activation were found only in the group of female smokers compared to female non-smokers.

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End Stage Renal Disease by patients with malignancy – ethical problems

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Abstract

The problem of co-occurrence of kidney failure, as well as ESRD and malignancy is relatively often and brings a significant therapeutical and moral challenge. The ethical basis of our consideration are "Evangelium Vitae" by John Paul II and "Declaration Jura et Bona".

The fundamental choice is whether to start and/or continue the kidney replacement treatment. We present 3 algorithms for the most typical situations.

The first ethical postulate in our considerations is that patients with the malignancy of good prognosis should not be denied of any treatment chance and should be dialysed as any other patient.

In the situation of patients with neoplastic disease with bad prognosis and ESRD, the question of 'withholding or withdrawing' dialysis is essentially part of a fundamental question, what should be the ultimate goal of medicine?

There is no doubt that the person most authorized to take a decision in such a situation is the patient provided it is a conscious decision based on full information. Therefore any decision to cease treatment, even submitted at the public notary, should be verified as long as a conscious contact with a patient is possible.

In the situation of continued lack of logical contact with the patient who has not left any clear disposition for such circumstances we must take the decision based on their benefit. It is more than desired that the decision acquires the approval of the patient's family but in the situation when it is not possible the doctor decide. In the doubtful cases we should take decisions "towards life".

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The progress in medicine is one of the most important though not the only reason which makes people's life longer. In the case of kidney replacement treatment it means that in the countries of highly developed medicine it is possible to use the treatment for all patients with kidney malfunctions. However, no one can discuss dialysis without considering the financial implications of such a decision [1].

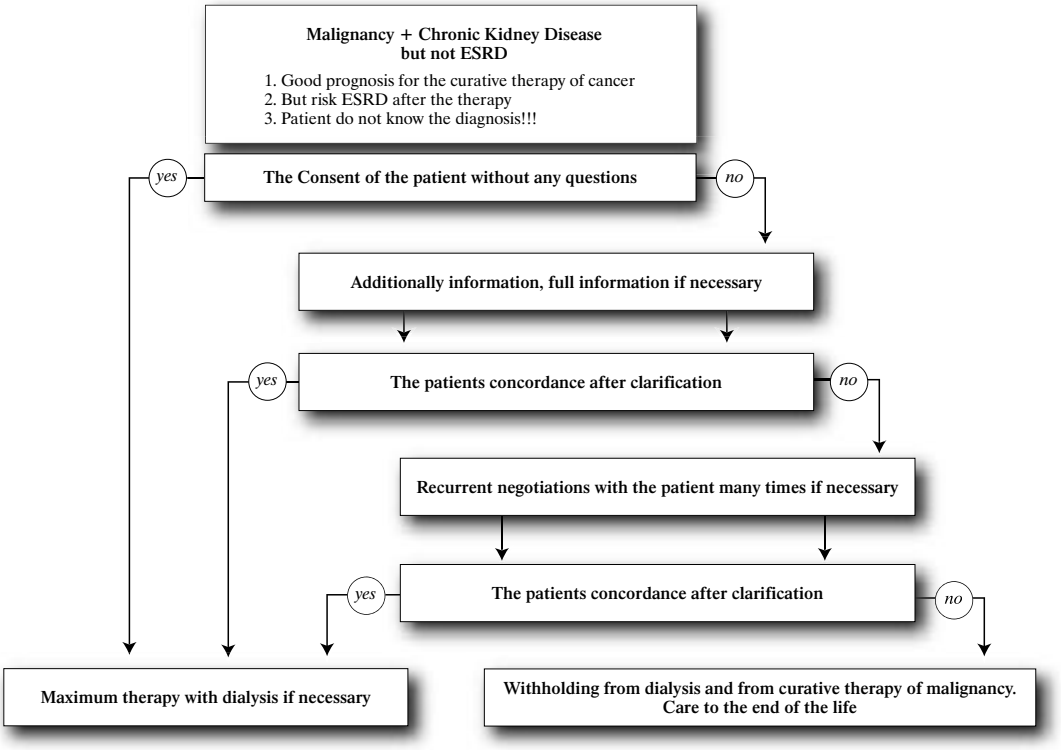
The dialysis treatment ceased to be something unusual or using the bioethical term, it ceased to be an extraordinary means. It does not mean that patients with kidney malfunctions ceased to die (as all other people), however the direct cause of death was not or at least should not be kidney disease.

Patients with End Stage Renal Disease (ESRD) have increased risk of many diseases including cancer. In the year 2002 cancers as the cause of death among German patients with dialysis (9%) occurred more rarely than in general population [2]. The distribution of tumor types resembles the pattern seen after transplantation. The excess risk can largely be ascribed to effects of underlying renal or urinary tract disease, or of loss of renal function, on the kidney and bladder, and to increased susceptibility to viral carcinogenesis. The relative risk, which is especially high in younger patients, gradually diminishes with age [3]. But in the older patients mortality is mostly associated with the presence of cancer ($p=0.053$) [4].

The problem of co-occurrence of kidney failure, as well as ESRD and malignancy is relatively often and brings a significant therapeutical challenge. In the face of dramatically circumstances and a necessity to make difficult moral choices it has its ethical dimension which is the subject of the present analysis.

The fundamental choice is whether to start and/or continue the kidney replacement treatment, most often dialysis in patients with diagnosed tumors or qualified to kidney transplantation in patients of high risk of cancer. Apart from somatomedic aspects one has to take into consideration the psychomedic aspects. The diagnosis

Figure 1. **Ethical considerations in case malignancy and Chronic Kidney Disease but not ESRD**



of cancer, and what should be stressed, regardless of the type, advancement and real prognosis is regarded as a death sentence.

Despite the awareness of doctors as for the existence of many other diseases such as, for example, circulatory insufficiency, which is a worse prognosis, cancer diagnosis similarly to HIV (but not viral hepatitis) causes psychological and social stigmatisation. It is easy, in such situations come to medically groundless resignation from various forms of intensive treatments, in order "not to prolong suffering" which in fact do not appear or at least not in the intensity that would make a real problem.

Therefore the first ethical postulate in our considerations is that patients with the malignancy of good prognosis should not be denied of any treatment chance and should be dialysed as any other patient. The only restriction is the period of awaiting for registering them as candidates for the kidney transplant. The decision is a little more difficult when the prognosis seems relatively positive but the cancer treatment method might cause permanent renal damage and/or worsening of their renal functioning including ESRD. A model example could be the cancer of the only kidney, but a similar case is the situation of a patient in the pre-dialysis situation who is to undergo intensive chemical therapy with nephrotoxic medicine applied. Following the principle of greater benefit for the patient it seems reasonable to propose "maximal therapy", that is cancer treatment and apparent dialysis programme. The decision, of course, is not with the patient.

Additionally, especially in Poland and perhaps in most traditionally Catholic countries, there may be a problem of informing the patient about the nature of their illness, which may in turn violate their "right to unawareness". The best solution seems to be the principle described in article 17 of the Medical Ethics Code: "In the case of unfavourable prognosis, the patient should be informed about it tactfully and with caution. The news about the diagnosis and bad prognosis may not be revealed to the patient only in the case if a doctor is fully convinced that the fact of revealing will cause suffering or other unfavourable consequences for the patient's health; however the doctor is obliged to full information at the clear demand from the patient"[5].

At the same time, we think that in the situation when the patient is not aware of the nature of their disease and a real risk and could make a wrong decision, from the medical point of view (refusal to undergo therapy), they should definitely be informed as to make their decision concerning life and death based on the truth. And again the patient should be informed about it tactfully and with caution [6] (*Fig. 1*).

The really difficult problem to be solved is the situation of patients with neoplastic disease with bad prognosis and ESRD. Those patients can be insured neither cure nor life of high quality and/or without suffering, nor even significant life prolongation. Thus we face the situation described in "Evangelium Vitae" by John Paul II from 1995 [6]: "In such situations when death is imminent and inevitable one may, in accord with one's conscience, resign from procedures which would only cause temporary and painful life prolongation, however the ordinary therapy required in such situations should not be ceased". "Evangelium Vitae" refers to previous declaration

"Jura et Bona" from 1980 [7]: "When death is imminent and cannot be avoided despite using available means, one is free in one's conscience to decide to cease treatment which may result only in uncertain and painful life prolongation". Referring to these documents which are important to Catholics (who are the majority of both patients and doctors in Poland) but also significant to people of other denominations and religions and even for non-believers, we must mention fragments which are significant for our professional responsibility. It means indicating the difference in practice between cessation of persistent therapy which is our responsibility for a dying patient and passive euthanasia which is not an act of true compassion. Knowing the difference between the two is extremely difficult in practice despite relatively precise definitions.

According to "Evangelium Vitae" euthanasia in the strict sense is understood to be an action or omission which of itself and by intention causes death, with the purpose of eliminating all suffering. "Euthanasia's terms of reference, therefore, are to be found in the intention of the will and in the methods used". [6]. However, euthanasia must be distinguished from the decision to forego the so-called too "aggressive medical treatment", in other words, medical procedures which no longer correspond to the real situation of the patient, either because they are by now disproportionate to any expected results or because they impose an excessive burden on the patient and his family. In such situations, when death is clearly imminent and inevitable, one can in conscience "refuse forms of treatment that would only secure a precarious and burdensome prolongation of life, so long as the normal care due to the sick person in similar cases is not interrupted" [6]. Certainly there is a moral obligation to care for oneself and to allow oneself to be cared for, but this duty must take account of concrete circumstances. It needs to be determined whether the means of treatment available are objectively proportionate to the prospects for improvement. To forego extraordinary or disproportionate means is not the equivalent of suicide or euthanasia; it rather expresses acceptance of the human condition in the face of death [6].

It is also permitted, with the patient's consent, to interrupt these means, where the results fall short of expectations. But for such a decision to be made, account will have to be taken of the reasonable wishes of the patient and the patient's family, as also of the advice of the doctors who are specially competent in the matter [7].

Doctors who are specially competent in the matter (is that us?) may in particular judge when:

– the investment in instruments and personnel is disproportionate to the results foreseen and

– the techniques applied impose on the patient strain or suffering out of proportion with the benefits which he or she may gain from such techniques [7].

Further consideration will focus on such judgment with reference to patients with ESRD and advanced neoplastic disease. For these patients the question of 'withholding or withdrawing' dialysis is essentially part of a much more important and fundamental question, namely: what should be the ultimate goal of medicine and health care workers [8].

There is no doubt that the person most authorized to take a decision in such a situation is the patient provided it is a conscious decision based on full information. This requirement is not always easy to fulfill, firstly because of the earlier described difficulties connected with giving the patient unfavorable news and a possible negative impact of such news on the last days of their life; secondly because of difficulties faced by any healthy man, also a doctor, to picture oneself realistically in the terminal condition. Therefore any decision to cease treatment, even submitted at the public notary, should be verified as long as a conscious contact with a patient is possible. It is very important because death after withdrawing from dialysis does not most frequently occur immediately.

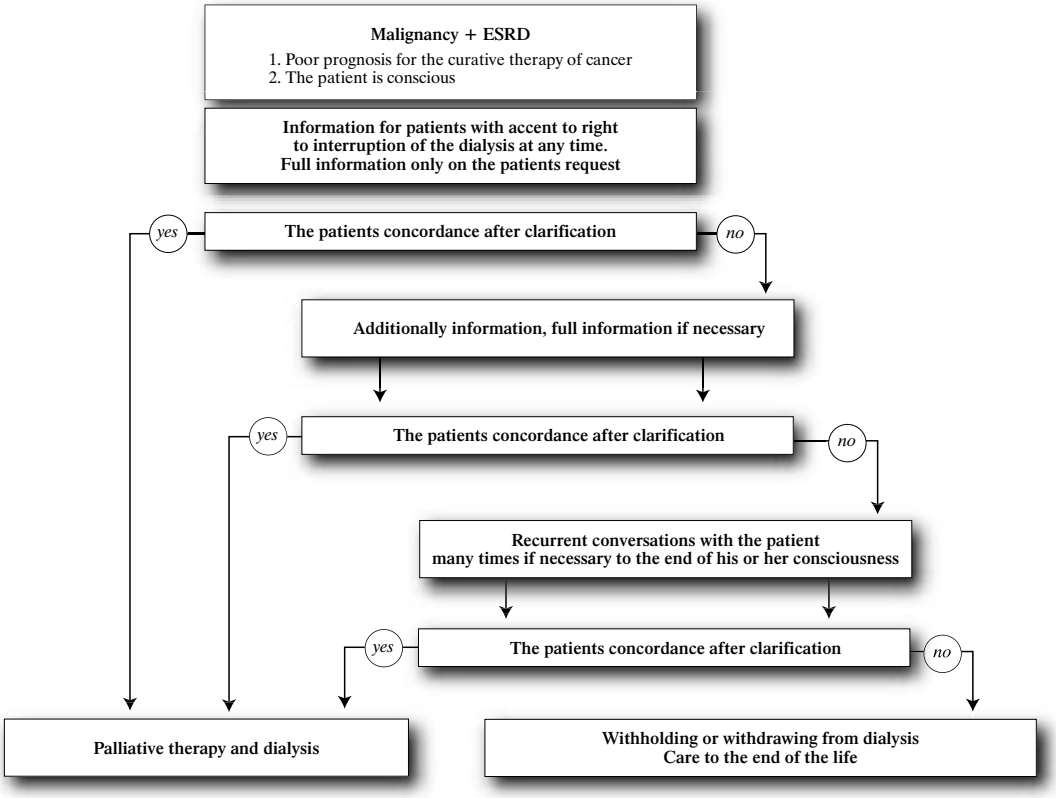
In recent observation from France after the last session of dialysis, the mean survival rate was 8.5 ± 4.8 days (median 7 days, range 4-21 days) [9]. In other opinion if patients died <3 days after withdrawing from dialysis is unlikely that withdrawing from dialysis was the principal cause of death [10].

In fact only 10% [9] to 14% [11] patients decided themselves to stop treatment. Nobody or very few patient's give advance directives [9,12].

The decision to withhold is made mainly by the nephrology team. In the USA in the early 1970s the physician initiated the decision in 66 percent of all patients; in the early 1980s this figure had decreased to 30 percent [13]. In other countries contemporary the situation is similar to USA in 1970s. In recent survey of Jolly and co. the physician initiated the decision in 86% [11]. But the role of family members is growing also in Poland. This may be some support for the treatment team, however, it imposes on the doctor a difficult responsibility to verify the true intentions of the family. Finally in the situation of continued lack of logical contact with the patient who has not left any clear disposition for such circumstances we must take the decision based on their benefit. In this difficult decision we may sometimes be directed by the so-called assumed will of the patient, that is the analysis of their opinions and choices made in their life. It is more than desired that the decision acquires the approval of the patient's family but in the situation when it is not possible the doctor must remember about his responsibility, first of all a moral one for the patient but also legal. One has to remember that our actions undergo legal judgement even after many years as well as ethical judgement. That is why there is a need for legal regulations which will indicate proper court (bioethical committee?) which will solve any possible disagreements between doctors and family of the terminal ill patient. It would certainly require very prompt actions which are unlikely in the present state of organisation of the legal system in Poland. It should be clearly emphasized that our decision cannot be determined by earlier decisions especially the beginning of dialysis therapy. In the ethical sense the distinction, common present in literature, between withholding and withdrawing from dialysis is of little significance. It is very important because doubtful cases we should take decisions "towards life", especially being aware that this solution, if mistaken, is not irrevocable. In practice it is known that doctors refuse to start dialyses more often than to withdraw from them because it is less burdensome for them from the psychological point of view [14] (*Fig. 2*).

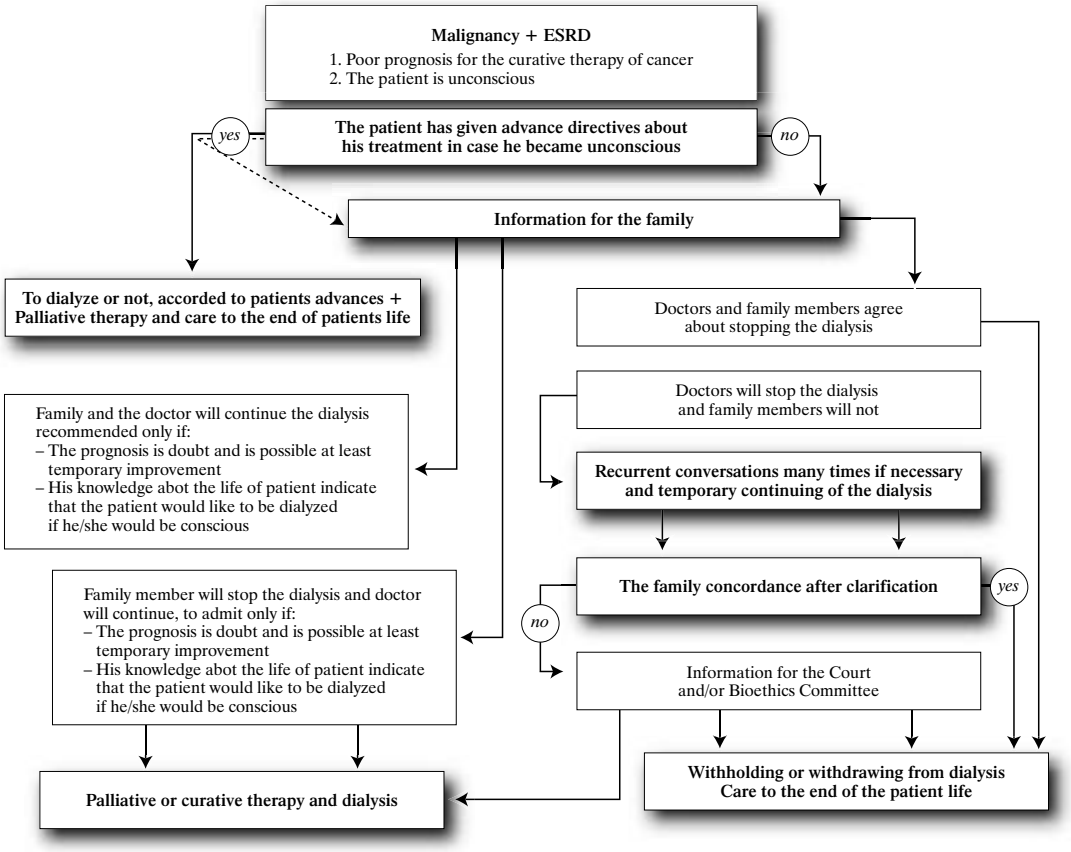
Finally, when the prognosis is definitely bad, contact with the patient is impossible to establish and there are no previous decisions from the patient for that predicament and the patient's life

Figure 2. **Ethical considerations case of malignancy and ESRD if the prognosis is poor and the patient is conscious**



would be continuous suffering we must have courage to withdraw from dialysis and focus on palliative treatment, care and nursing for the days of their life (*Fig. 3*). This most significant decision should be taken by at least two doctors, with at least of them being a specialist in nephrology. The presence of nephrologist favours decisions to carry on dialysis. American experience indicates that family doctors and even internists more easily disqualify patients from kidney replacement treatment without the need to consult the nephrologists [15]. Patients who were predominantly cared for by a general internist were more likely to be referred late to a nephrologist compared with those cared for by a family or primary care practitioner (P=0.002) or another subspecialist (P=0.019) [16]. Delayed referral was highly associated with older age (P<0.001), race other than white or black (P=0.002), and the absence of certain comorbidities: hypertension (P<0.001), coronary artery disease (P<0.001), malignancy (P=0.005) and diabetes (P=0.02). Associations of late referral with male sex (P=0.07) and lower socioeconomic status (P=0.09) were of borderline significance. [16]. Neoplasm's constitute the second in frequency cause of death in general population. The situation was similar among dialysed patients, but recently there was a retrospective report by Birmele et al. The study concerned morbidity in 1436 dialysed patients in France in 2001. In this survey death after withdrawing from dialysis was the most common cause of death (20%) comparable with cardiovascular diseases (18%) and 3 fold greater then cancer (6%) [9]. Cancer was more frequent in the withdrawing group 15% vs continuing patients (7%), but in this small cohort this difference was not significant (p=0.15) [9]. But in the medical record withdrawal from dialysis was equal with cancer 13 vs 12 as the cause of death [9]. But only a further analysis of files showed that in reality the number was not 13 but 40. It should be emphasized that in a patient with a number of disorders as the majority of patients with ESRD and cancer assessing the real cause of death is not easy. Withdrawing from dialysis causes the patient's death after approximately 2 weeks (on average after about 10 days sometimes after a month or longer) [17]. It is frequently assumed that it is impossible that death is a result of withdrawal from dialysis if a patient dies within 3 days in the case of hemodialysis and 7 days of peritoneal dialysis [9,10]. That is why it seems that withdrawal from dialysis is less frequently mentioned as the cause of death than it is the case in reality. In Poland according to few published data the percentage is significantly lower (<1%). According to surveys conducted by our department approximately 1/3 of Polish nephrologists have encountered the problem for the past 5 years but in most cases it concerned withholding rather than withdrawing from treatment [12]. In the recent analysis from USA 26% of patients with ESRD dialysis was stopped before death, but 30% of these patients died <3 days and only in 4% of these patients uremia was indicated as the cause of death. [10]. We were not able to differentiate patients terminating therapy from those continuing treatment on the basis of age or co-morbidity, suggesting that subjective patient perception of their condition is a critical factor in stopping dialysis [18]. It is concluded that beside a patient's individual refusal, late

Figure 3. **Ethical considerations the case of malignancy and ESRD if the prognosis is poor and the patient is unconscious**



referral, social isolation, low functional capacity, and diabetes may have oriented medical decision toward withholding dialysis in a significant proportion of pre-ESRD octogenarians [11], after earlier examination cancer, malnutrition, catabolism, and "dissatisfaction with life" were important associations with the decision to withdraw [19]. But it is not the tumors but a Vascular nephropathy which is the principal disease predicting withdrawal from dialysis; the main precipitating cause is mental incapacity [20]. The physicians with a background in bioethics have a higher rate of withdrawal and/or withholding from dialysis than those who did not have these specific skills [21]. Those doctors probably understand slightly differently their responsibilities and the patient's rights. Perhaps they also understand more deeply the Hippocrates oath where instead of Latin translation of "primum non nocere" – do no harm, they use the original Greek text with the positive expression: ophelein, which in fact means to benefit [8]. Taking into consideration the patient's benefit we present below the proposal of the management scheme in case of ethical doubts in patients with ESRD and cancer.

Final recommendations

1) The decision to withdraw from dialysis as crucial for life or death should be taken by at least two doctors including a nephrologis. It is advisable to discuss the decision at the meeting of the caring team.

2) The patient's will or in case when he/she is unconscious, the previously expressed wish in a written form concerning his/her treatment in such circumstances is the most important factor while taking the decision to continue or withdraw from treatment.

3) Close relatives (or people indicated by the patient and/or people who in our opinion act for the benefit of the patient) should be informed about the doubts and purposefulness of further treatment and together try to come to a common agreement. But the final decision is with the doctors.

4) Before making the decision about continuing or withdrawing from dialysis, consultations with priests, who have bioethical knowledge, lawyers or philosophers.

5) The fact of making such a decision should be described in the patient' file – with due respect to the principles of medical confidentiality. It is advisable to have a uniform model form developed by the Ministry of Health.

6) There is a need for legal regulations concerning the protection of the patient but also the legal protection for those taking part in the decision making process concerning continuing or discontinuing dialysis. The regulations should include the offices and institutions which have the right and obligation to make the decision in the situation of conflict and procedures suitable for hospital reality.

7) In doubtful cases the decisions should be directed towards life also because the contrary actions cannot be corrected.

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Safety of various methods of intensive insulin therapy in hospital condition assessed by hypoglycaemic episodes detected with the use of continuous glucose monitoring system

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Abstract

Purpose: The aim of the study was to determine the safety of three intensive insulin therapy methods: multiple daily insulin injections (MDI), continuous subcutaneous insulin infusion (CSII) and continuous intravenous insulin infusion (IVII) used in poorly controlled type 2 diabetic patients in hospital condition. The safety of these intensive insulin therapy methods was measured by the assessment of number and duration of symptomatic and symptomfree hypoglycaemic events with use of Continuous Glucose Monitoring System (CGMS, Medtronic MiniMed).

Material and methods: The study comprised 90 type 2 diabetic patients treated with conventional insulin therapy based on a twice daily injections with mean glucose profile values >14 mmol/l. The patients were randomized into three groups according to the method of insulin treatment. The first group was treated with MDI, the second group with CSII and the third with IVII. The glucose monitoring with the use of CGMS lasted 48 hours and was conducted on the second and on the third day of intensive insulin therapy. Glucose level below 3.5 mmol/l were recognized as hypoglycaemic episode. Intensive insulin treatment was continued until "near normoglycaemia" (glucose levels 4.5-10.0 mmol/l) was achieved and then conventional insulin therapy was readministrated.

Results: Mean number of symptomatic hypoglycaemic events detected with CGMS was two times higher for MDI than for IVII (p=0.04) and for CSII (p=0.04). Number of symptomfree hypoglycaemic events detected with CGMS was higher for MDI than for IVII and CSII, but the differences were insignificant (NS). Mean duration of one symptomfree hypoglycaemic event detected with CGMS was longer in MDI than in CSII (p=0.02) and IVII (p=0.03). It was not observed significant differences in mean duration of one symptomatic hypoglycaemic episode between studied groups (NS).

Conclusions: The results of study suggest that CSII and IVII treatment is associated with essentially lower number of symptomatic hypoglycaemic events and shorter mean duration of one symptomfree hypoglycaemic event than MDI.

Key words: multiple daily insulin injections, continuous subcutaneous insulin infusion, continuous intravenous insulin infusion, continuous glucose monitoring system, hypoglycaemia.

Introduction

Poorly controlled type 2 diabetic patients treated with hypoglycaemic oral drugs or conventional insulin therapy often require short-term intensive insulin treatment as part of a planned attempt to improve glycaemic control. It comprises multiple daily insulin injections (MDI), continuous subcutaneous insulin infusion (CSII) and continuous intravenous insulin infusion (IVII). MDI, CSII and IVII can in the best way imitate diurnal physiological rhythm of insulin secretion as compared to other methods of insulin treatment and enable adjusting individual dosage of insulin according to patient's physical condition, his activity and meals. Side-effects of insulin treatment, including MDI, CSII and IVII are hypoglycaemic events, which are an obvious life hazard. Symptomfree hypoglycaemic episodes – not recognized by patients are particularly dangerous.

Glycaemia monitoring is an essential element of successful diabetes-treatment. The course of diabetes is characterised by fluctuation of glycaemia and it is obvious, that sporadical measurements of glycaemia with glucose meters can not assure the proper treating. It seems, that the optimal solution of this problem can be continuous glucose monitoring systems. Continuous Glucose Monitoring System (CGMS, Medtronic MiniMed) is

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Table 1. **Characteristics of subjects**

	group 1 (MDI)	group 2 (CSII)	group 3 (IVII)	p
Number of subjects	30	30	30	NS
Sex F/M	20/10	22/8	19/11	NS
Age [years] x±SD	60.1±7.2	57.4±6.6	58.9±6.4	NS
BMI [kg/m^2] x±SD	27.1±2.6	27.5±2.4	27.5±1.9	NS
Duration of diabetes [years] x±SD	6.2±2.6	5.5±2.8	5.6±3.0	NS
Duration of insulin therapy [years] x±SD	2.9±1.9	2.6±1.7	3.1±2.4	NS
Mean daily insulin dose [I.U./kg] x±SD	0.72±1.1	0.81±0.8	0.70±1.4	NS
Blood glucose profile one day before short-term intensive insulin therapy introduction [mmol/l] x±SD	13.8±3.0	13.0±3.3	14.2±1.4	NS
HbA1c [%] x±SD	8.0±2.1	7.9±1.7	7.8±1.5	NS

one of such devices. This method enables strict assessment of glucose profile. It draws the glycaemia curve according to 288 glucose measurements per 24 hours [1-6].

The aim of the study was to determine the safety of short-term intensive insulin therapy methods: MDI, CSII and IVII in poorly controlled type 2 diabetic patients. The safety was measured by the number and duration of symptomatic and symptomfree hypoglycaemic events with use of CGMS.

Material and methods

The study comprised 90 type 2 diabetic patients with daily glucose profile (mean glucose value determined on base of four measurements during one day before breakfest, dinner and supper at 8.00, 13.00, 18.30 h respectively and 90 min after supper at 20.00 h) >14 mmol/l in the last 7 days before admission to Department of Diabetology and Metabolic Diseases at Medical University of Łódź. There were clinical grounds for their admission. Including criteria were the following: age between 50-70, at least 0.5 year of insulin treatment based on a twice daily injections of insulin mix 30/70 (Humulin M3 – Eli Lilly, Mixtard M30 – Novo Nordisk, Gensulin M30 – Bioton), body mass index (BMI) 25-30 kg/m^2. Patients with advanced neuropathy, microangiopathy, and macroangiopathy, with liver diseases and other serious general illnesses, psychiatric problems, infectious diseases, tumours, alcoholism, pregnant or breast feeding women were excluded from this study as well as patients taking corticosteroids, thyroid hormones, neuroleptics and thiazyd diuretics.

The study group consisted of 61 females and 29 males with mean age 58.8+/-7.2 yrs, mean BMI 27.4+/-2.6 kg/m^2, mean diabetes history 5.8+/-3.0 yrs and mean insulin therapy periods 2.9+/-2.0 yrs. Mean daily glucose values during the first 3 days of hospitalisation before starting the study was mean 13.7+/-3.3 mmol/l.

The patients were randomized into three groups according to the method of intensive insulin therapy. The first group was treated with MDI, the second group with CSII and the third with IVII. The groups didn't differ significantly regarding: their age, BMI, HbA1c, duration of diabetes and insulin therapy, clinical course of diabetes type 2, daily profile of glycaemia during the first 3 days of hospitalisation. Detailed characteristics of the groups are presented in the *Tab. 1*.

All patients provided written informed consent. The study was approved by the Ethics Committee for Studies in Subject at the Medical University of Łódź and was conducted in accordance with the Declaration of Helsinki and good clinical practice guidelines.

During the first 3 days of hospitalisation conventional insulin therapy was maintained and insulin doses were not modified, and the patients were adviced on dieting. Every patient was applied isocaloric diet, containing 200-250 g of carbohydrates per day, divided into 3 main meals in proportion: 20%-40%-40%, which were consumed at 8.00 am, 1.00 pm, 6.30 pm respectively. Ten measurements of blood glucose with glucose meters (MediSense Precision Q.I.D, Abbott) were performed daily since the first day of hospitalisation. Glucose measurements were taken at 8.00 am, 9.30 am, 1.00 pm, 2.30 pm, 4.00 pm, 6.30 pm, 8.00 pm, 10.00 pm, 1.00 am, 5.00 am. On the base of these glucose values daily glucose profile was determined. On the fourth day of hospitalisation at 7.00 am an intensive insulin therapy was introduced. The glucose monitoring with the use of CGMS was introduced on the second day of intensive insulin therapy.

MDI was based on the application of short-term insulin analogue LysPro (Eli Lilly) 3 times a day before every main meal and NPH insulin (Eli Lilly) at 10.00 pm. Preliminary insulin dose, applied on the first day of MDI was estimated according to daily insulin dosage, that patient had needed before the hospitalisation. 70% of daily insulin dosage was applied in form of prandial boluses in appropriate ratio: 30% before breakfast, 20% before dinner and 20% before supper and the rest – 30% of daily insulin dosage was applied at 10.00 pm [7].

CSII and IVII were applied with short-term insulin analogue LysPro (Eli Lilly). CSII was applied with the use of personal subcutaneous pump (508, Medtronic MiniMed) and IVII with the use of intravenous pump (Duet standard 50, Kwapisz). Initial insulin flow was estimated according to the algorithm proposed by Ruxer J. et al. [8]. CSII and IVII comprise three ninety minutes "prandial insulin square-boluses", which are started at the beginning of breakfast, dinner and supper at 7.00 am, 1.00 pm, 7.00 pm respectively and four basic insulin infusions lasted from 8.30 am till dinner time (1.00 pm) and from 2.30 pm till supper (7.00 pm), from 8.30 pm till 1.00 am and from 1.00 am till breakfast (7.00 am). Initial insulin dosage both for basic flow and prandial boluses was established according to

***Table 2*. Comparison of daily blood glucose profile before and on the last day of short-term intensive insulin therapy**

Subjects	Mean blood glucose (mmol/l ±SD)		p
MDI	0	13.8±3.0	p < 0.001
	1	7.9±2.1	
CSII	0	13.0±3.3	p < 0.001
	1	6.6±2.2	
IVII	0	14.2±1.4	p < 0.001
	1	7.2±1.9	

0 – Daily blood glucose profile determined one day before short-term intensive insulin therapy; 1 – Daily blood glucose profile determined on the last day of short-term intensive insulin therapy

***Table 3*. Mean number of hypoglycaemic episodes detected with CGMS during 48 h observation period**

	Mean number of symptomatic hypoglycaemic episodes detected with CGMS	Mean number of symptom-free hypoglycaemic episodes detected with CGMS
	mean±SD	mean±SD
MDI	2.0±0.1	0.85±0.1
CSII	1.15±0.14	0.65±0.12
IVII	1.15±0.12	0.55±0.16
MDI vs CSII	**p=0.04**	NS
MDI vs IVII	**p=0.04**	NS
CSII vs IVII	NS	NS

the patient weight. Insulin doses were established in 24 hours advance and since the second day of CSII and IVII were based on glucose profile obtained the previous day. If hypoglycaemia happened CSII and IVII were stopped, till glucose level reached 5.5 mmol/l [8]. If there were clinical symptoms of hypoglycaemia 40% glucose solution was applied intravenously.

All the actions connected with IVII, CSII treatment, glucose measurement with the use of glucose meters and CGMS service were carried out by professional medical staff. The glucose monitoring with the use of CGMS lasted 48 hours and started on the second day of intensive insulin therapy. Glucose level below 3.5 mmol/l were recognized as hypoglycaemic episode. Intensive insulin treatment was continued until "near normoglycaemia" (glucose levels 4.5-10.0 mmol/l) was achieved, then intensive insulin treatment was discontinued and insulin treatment based on a twice daily injections of insulin mix 30/70 was applied.

Presented data are means ±SD. Characteristics of groups, duration of hypoglycaemia and number of hypoglycaemic episodes were compared between these groups using ANOVA. All reported p values <0.05 were considered statistically significant. STATISTICA was used for all analyses.

Results

It was observed, that short-term methods of intensive insulin therapy: MDI, CSII and IVII are associated with significantly reduction of glucose values in all groups (*Tab. 2*).

Table 4. Mean duration of one hypoglycaemic episode (minute) detected with CGMS during 48 h observation period

	Mean duration of one symptomatic hypoglycaemic episode detected with CGMS	Mean duration of symptom-free hypoglycaemic episode detected with CGMS
	mean±SD	mean±SD
MDI	47±13	36±17
CSII	46±16	20±18
IVII	36±11	26±14
MDI vs CSII	NS	**p=0.02**
MDI vs IVII	NS	**p=0.03**
CSII vs IVII	NS	NS

***Table 5*. Comparison of mean daily insulin dose [IU/kg] x±SD on the second and on the thirth day of intensive insulin therapy introduction between study groups**

Time points	MDI	CSII	IVII	p
2nd day	0.77±0.9	0.74±0.5	0.71±0.6	NS
3rd day	0.82±0.2	0.79±0.4	0.84±0.3	NS

Mean number of symptomatic hypoglycaemic events detected with CGMS was almost two times higher for MDI than for IVII (p=0.04) and CSII (p=0.04) (*Tab. 3*).

Mean number of symptomfree hypoglycaemic events detected with CGMS was higher for MDI than for IVII and CSII, but the differences were insignificant (NS) (*Tab. 3*).

Mean duration of one symptomfree hypoglycaemic event detected with CGMS was longer in MDI than for CSII (p=0.02) and for IVII (p=0.03) (*Tab. 4*).

There was no significant differences in mean duration of one symptomatic hypoglycaemic episode between studied groups (NS) (*Tab. 4*).

There was no significant differences in mean daily insulin dose on the second (NS) and on the third day of intensive insulin therapy between study groups (NS) (*Tab. 5*).

Mean duration for MDI therapy was 6.0±2.0 days, for CSII 5.0±3.0 days and for IVII 5.5±2.5 days (NS).

Discussion

This study determined the safety of MDI, CSII and IVII in poorly controlled type 2 diabetic patients by evaluation of number and duration of symptomatic and symptomfree hypoglycaemic events. According to analysis of glycaemia values measured with use of CGMS, it seems that the MDI is the most dangerous method of short-term intensive insulin therapy compared with CSII and IVII. The safety of MDI, CSII and IVII in poorly controlled type 2 diabetes assessed by hypoglycaemic episodes with use of CGMS has not been analyzed either. Furthermore, the duration of hypoglycaemic episodes have not been used for assessing safety of intensive insulin therapy.

It is worth to introduce pioneering study DCCT. On the base of which it was affirmed that 36% of hypoglycaemic events were

symptomless, out of which 55% happened at night. There were no differences observed in number of hypoglycaemic events between CSII and MDI in DCCT [9].

The study carried out by Bode et al. compared two long-term intensive insulin therapy methods: MDI and CSII. 225 patients were treated with MDI for 12 months and 138 serious hypoglycaemic events were observed during this period. After that time the treatment was changed into CSII. Twenty two serious hypoglycaemic events were observed in the second year of study. It was concluded, that CSII caused 6-fold less serious hypoglycaemic events than MDI [10].

In Boland's study number of hypoglycaemic events during the intensive insulin therapy: MDI and CSII were estimated. Serious hypoglycaemic events were detected twice often in MDI than in CSII [11].

According to Eichner's study, long-term CSII causes less number of serious hypoglycaemic events than MDI. Twenty two serious hypoglycaemic events in patients treated with CSII and 273 serious hypoglycaemic events in patients treated with MDI were detected during 1 study year [12].

In Bendtson's study frequency of nocturnal hypoglycaemic events in MDI and CSII type 1 diabetic patients with use of CGMS were detected. Glucose level beneath 3.5 mmol/l was recognized as hypoglycaemic event. Nocturnal hypoglycaemic events were detected in 30% of the patients treated with MDI and 44% treated with CSII. Mean duration of symptomfree episode in CSII patients was 2 hours while in MDI patients 4 hours [13].

Chase in his study measured glycaemia profile in 11 type 1 diabetic patients for 30 days with use of CGMS. Only every sixth hypoglycaemia was detected with clinical symptoms. Definitely majority of hypoglycaemic events were symptomless and only CGMS made the hypoglycaemia detection possible [14].

Pańkowska obtained similar results suggesting, that CGMS is a very important way of detection of hypoglycaemic events. Thirty three type 1 diabetic patients were enrolled into the study. Through 3 to 4 days of the study hypoglycaemic events detected with CGMS were observed in 78% of the patients and only every fourth event was detected by glucose meter [15].

Conclusions

The results strongly suggest, that:

1. CSII and IVII are more safe methods of short-term intensive insulin therapy assessed by hypoglycaemic episodes than MDI.

2. CGMS can be very useful particularly in patients with tendency for hypoglycaemic events during poorly controlled diabetes. It significantly increases the safety of treatment through full hypoglycaemic event record.

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Mucosal gastrin cells and serum gastrin levels in children with *Helicobacter pylori* infection

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Abstract

Purpose: Impaired control of gastric juice secretion is observed in chronic gastritis due to *Helicobacter pylori* (*H. pylori*) infection. G cells are stimulated by such cytokines as tumor necrosis factor (TNF-alpha), interferon gamma (IFN-gamma) and interleukin-8 (IL-8). The number of D cells producing somatostatin decreases simultaneously. An increase in gastrin levels could also depend on alkalization in G cell environment caused by bacterial urease. The aim of the study was to evaluate G cell counts in the antrum and gastrin levels in the serum of children with *H. pylori* infection and after bacterium eradication.

Material and methods: The study was performed in 106 patients. Children were divided into 3 groups with regard to the presence and course of *H. pylori* infection. Fifty nine children (55.7%) had chronic gastritis in the course of *H. pylori* infection with a positive titre of antibodies in IgG class against *H. pylori*; 29 children (27.3%) with past *H. pylori* infection, without bacterium colonization and gastritis but with a positive titre of antibodies in IgG class against *H. pylori*; 18 children (17%) with functional disorders of the gastrointestinal tract but without *H. pylori* infection.

Results: The quantitative analysis of gastrin cells in the antral mucosa of children performed by immunohistochemical method showed the highest gastrin cell count in group I with *H. pylori* infection (112.1±58.9 cell/mm^2) and in group II with past *H. pylori* infection (105.3±73.1 cell/mm^2). The serum gastrin level (92.9±41.6 μU/ml) was the highest in children with *H. pylori* infection. In controls, it was 70.0±15.3 μU/ml and could be compared to the results of children with past *H. pylori* infection.

Conclusions:

1. The *H. pylori* infection plays a significant role in the stimulation of G cells increase and gastrin release in the blood serum in children.

2. The eradication of *H. pylori* infection is probably a main factor in gastric secretion down-regulation during gastritis in children.

Key words: gastrin, gastrin cell, *Helicobacter pylori*, children.

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Introduction

The disorders of gastric juice secretion occur in the course of *Helicobacter pylori* infection, which contributes to the damage of gastric mucosa induced by an increase in gastrin production by G cells and a decrease in somatostatin secretion by D cells. Gastrin acting on the lining cells enhances the secretion of hydrochloric acid and increases the effect of histamine and acetylcholine. Few probable mechanisms are taken into consideration in the pathogenesis of increased gastrin secretion: antral mucosa inflammatory state per se (via IFN-gamma, TNF-alpha and IL-8 activity) activates the gastrin release by G cells, the antrum region alkalization with amonia stimulates directly G cells and increased leptin release induced by proinflammatory cytokines plays an important role in the control of gastrin production [1-3]. The decreased production and release of somatostatin play also an important role in this process. This is caused by a decreased count of D cells in the antrum, the activation of H_3 receptors on D cells due to N-alpha-metylhistamine production by *Helicobacter pylori* [4-7]. Hypergastrinemia observed in the course of gastritis and duodenal ulceration is caused by the hyperactivity of G cells, their multiplication (mucosa regeneration disorders present in the course of chronic inflammation) and their increased counts (most significant in atrophic inflammation with large changes) [1]. Two latter mechanisms seem to play a more important role in adults due to histopathological

changes characteristic of an inflammatory process persisting for a long time.

After effective bacterium eradication, D cell counts increase significantly, G cell counts decrease in the antrum and gastrin levels decrease in the antrum [8,9].

The aim of the study was to evaluate antral G cell counts and gastrin levels in the serum of children infected with *H. pylori* infection and after bacterium eradication.

Materials and methods

The study was carried out in 106 children divided into 3 groups with regard to the course of *Helicobacter pylori* infection.

Group I – 59 children (55.7%) with chronic gastritis in the course of *Helicobacter pylori* infection with a positive titre of antibodies in IgG class against *H. pylor*i. The group consisted of 29 girls (49.2%) and 30 boys (50.8%). The age of children ranged from 2 to 18 years and the mean age was 12.2±4.6 years.

Group II – 29 children (27.3%) after past *Helicobacter pylori* infection, without bacterium colonization and gastritis but with a maintaining positive titre of *H. pylori* antibodies in IgG class. The group consisted of 14 girls (48.3%) and 15 boys (51.7%). The age of children ranged from 3 to 18 years and the mean age was 11.0±4.2 years.

Group III – 18 children (17%) with functional disorders of the gastrointestinal tract, without *H. pylori* infection and with normal IgG concentration against *Helicobacter pylori*. The group consisted of 12 girls (66.75%) and 6 boys (33.3%). The age of children ranged from 5 to 17 years and the mean age was 10.7±3.6 years.

Endoscopic and histological examinations of the upper gastrointestinal tract were performed in all children, due to chronic or recurring abdominal pains. Gastrin cells were counted in the antral mucosae and gastrin levels were measured in the serum. The endoscopic and histopathological evaluation was carried out basing on the Sydney System classification [10]. When estimating *H. pylori* colonization, the intensification of inflamed mucosa characterization and inflammation severity were expressed in a 4-grade scale (0-1-2-3), and gastritis activity was measured in quantity of infiltrating granulocytes. Two samples each were taken from the antrum and the sites changed pathologically. The samples were stained with hematoxyline and eosine. The Giemsa method was used to identify *H. pylori* infection. The quick urease test (CLO-test-*Helicobacter pylori*) was done using the kits of the Institute of Food and Nutrition in Warsaw.

Gastrin level was estimated using a radioimmunoenzymatic test (RIA test, CIS Bio International). The blood serum samples were prepared for the examination according to the manufacturer's instructions. The method was based on the assessment of gastrin stained with iodine 125 bound with anti-gastrin antibodies and its free fraction. In this method, the minimum reference limit equals 10 μU/ml.

Gastrin cells were counted using rabbit's monoclonal antibodies against human gastrin (Polyclonal Rabbit Anti-Human Gastrin, DAKO Cytomation, Denmark). The picture analysis on PC computer was done by means of Olympus BX50 light microscope and Lucia G (Nikon, Japon) programming. The concentration of particular cells was defined on 1 mm^2 of the sample. In children with gastritis and *H. pylori* infection, eradication treatment with three medications was applied according to the indications of the workshop of the Polish Association of Gastroenterology [11]. The rest of children were given appropriate pharmacological and diet treatment according to the ailments found and the abnormal results of the tests. Ethical approval for research was obtained from local Ethics Committee in the Medical University of Białystok (R-I-003/30/2002).

Statistical analysis

The following descriptive methods of statistics were used for each characteristic and group of patients: arithmetic mean ($\bar{x}$), median, mode, and scattering measure, measure of distribution, standard deviation, variance and the value of upper and lower quartile. Chi^2 and Kolmogorov-Smirnov tests were used to match the distribution of empirical data with normal distribution. Mann-Whitney U non-parametrical test was used to find significant differences in the parameters between groups, regarding differences at $p<0.05$ as statistically significant. Result correlation was calculated with the use of the Spearman correlation test. ROC (Receiver Operating Characteristic) curves were analyzed to evaluate the usability of diagnostic parameters. A computer program STATISTICA 5.0 in the version for Windows System was used to calculate the data.

Results

Endoscopic evaluation of gastric mucosa in children with *H. pylori* (group I) showed inflammatory changes in the antrum in 71.2% of children and in the corpus in 64.4% of the examined. In group II (after *H. pylori* eradication, with positive IgG against *H. pylori*), inflammatory changes in the antrum and corpus were found in 27.6% of children ($p<0.001$). Children in group III (control) with negative IgG against *H. pylori* had normal gastric mucosa in the antrum and corpus. When examining children with *H. pylori* (group I), according to the Sydney System, the severe degree of antral gastritis was found in 45.8% of the examined; moderate gastritis in 52.5% and mild gastritis in 1.7%.

No severe gastritis was found in children with *H. pylori* eradicated (group II). The moderate degree of antral gastritis was proved in 6.9% of children and mild gastritis in 13.8%. Normal antral mucosa was found in 79.3% of children in group II. In controls, mild gastritis was observed in 15.4% and normal antral mucosa was found in 84.6% of children.

The evaluation of antral gastritis degree using Chi^2 test proved the statistically significant correlation in the study groups ($p<0.001$).

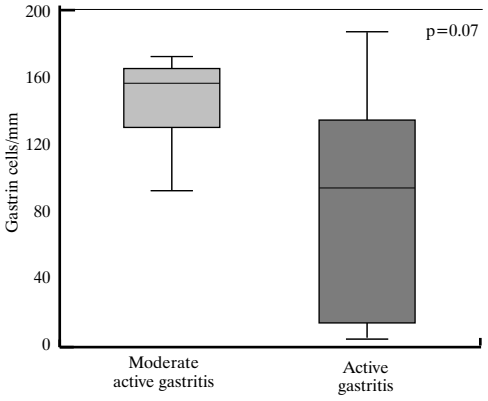
The analysis of antral gastritis activity indicated the most significant changes in children with *H. pylori* infection (group I). The severe active gastritis was found in 69.5% of children in this group; moderate active gastritis in 30.5% of the infected. No severe or moderate active gastritis was found in children with past infection after bacterium eradication (group II), but mild active gastritis was only in 20.7% of children in this group.

The analysis of antral gastritis activity using Chi^2 test proved

Table 1. **Gastrin cells in antral mucosa in children with *H. pylori* infection (I group), after eradication (II group) and in without infection *H. pylori* (III groups), Mann-Whitney U non-parametrical test**

Groups examined	Gastrin cells in antral mucosa/mm²								
	n	Min.	Max.	Arith. mean ($\bar{x}$)	Median (M)	Mode	Standard deviation (SD)	Lower quartile	Upper quartile
Group I	24	0	189	112.1	129.5	0	58.9	94.5	157
Group II	13	0	189	105.3	135.0	0	73.1	13.0	156
Group III	7	0	162	86.1	83.0	0	69.8	30.0	149

Figure 1. **Gastrin cell count and antral gastritis degree in children with *H. pylori* infection (group I), Mann-Whitney U non-parametrical test**



statistically significant results ($p<0.001$) in the groups examined. Histopathological evaluation of corpus gastritis was also performed basing on the Sydney System.

Corpus gastritis of mild and moderate degree (62.7% and 35.6% of the examined, respectively) predominated in children with *H. pylori* infection. Only 1.7% of children infected had corpus gastritis of the severe degree.

In children after *H. pylori* eradication (group II), normal corpus mucosa was observed in 82.1% of the examined, mild degree gastritis in 15.4% of children and moderate degree in 3.6%. In controls, only mild degree gastritis was observed in 15.4% and normal corpus mucosa was found in 84.6%. The results obtained in the histopathological evaluation of corpus mucosa changes were proved to be statistically significant ($p<0.001$).

Moderate active corpus gastritis was found in 55.9% and severe in 44.1% of children with *H. pylori* infection (group I). In children with past *H. pylori* infection after bacterium eradication (group II), mild active corpus gastritis was observed only in 17.9%. No moderate or severe activity was reported in this group. The results of histopathological evaluation of corpus inflammation were statistically different in each group ($p<0.001$).

The quantitative assessment of gastrin cells in the antral mucosa by immunohistochemical method showed the highest count in children with *H. pylori* infection (112.1 ± 58.9 cells/mm²) and in the group with past *H. pylori* infection (slightly lower 105.3 ± 73.1 cells/mm²). The results in both groups were higher in comparison with controls (86.1 ± 69.8 cells/mm²) (*Tab. 1*).

No statistically significant differences were found in gastrin cell counts in the antral mucosa with regard to the degree of antral gastritis ($p<0.07$). In children with *H. pylori* infection, the average count of gastrin cells in moderate degree gastritis was 145.4 ± 29.20 cells/mm². In the same group, the average count of gastrin cells equaled 88.36 ± 63.94 cells/mm² in severe degree gastritis (*Fig. 1*).

The average gastrin cell count in moderate active gastritis was 106.0 ± 77.18 cells/mm² and in severe active gastritis – 113.35 ± 57.01 cells/mm².

In children with *H. pylori* infection (group I), serum gastrin levels were highest and equaled 92.9 ± 41.6 μU/ml. They were also significantly elevated when compared to the levels of gastrin in group II with past *H. pylori* infection ($p<0.01$). In controls, serum gastrin levels were 70.0 ± 15.3 μU/ml and they could be compared to the levels in group II. *Tab. 2* presents the results of statistical analysis. In group I, a correlation between children's age and gastrin levels in the serum is non-linear and described by general formula $y=a+b/x$. This correlation is characterized by a decrease in gastrin levels in the serum together with an increase in the age of children (r -0.43, $p<0.001$).

A similar negative correlation (r -0.38) between age and gastrin levels was found in group II. It was also non-linear, described by a general formula $y=a+b/x$. This correlation is statistically significant ($p<0.005$) indicating a decrease in gastrin levels in the serum together with a increase in children's age. ROC analysis confirms a high usability of gastrin levels as a diagnostic parameter ($AUC=0.85\pm0.05$). The gastrin level of 61.38 μU/ml taken as a value criteria guarantees the highest accuracy of diagnosis. The sensitivity criteria equals 94.4% and specificity – 62.5% (*Fig. 2*).

Discussion

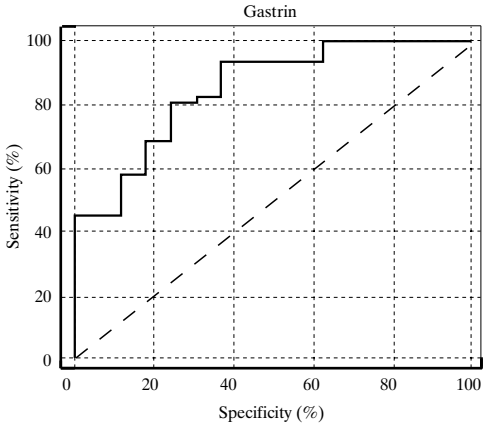
G cell counts in the antrum and gastrin levels in the blood serum were assessed in our study. A higher G cell count (mean value – 112.1 ± 58.9/mm²) was established in children with gastritis in the course of *H. pylori* infection in comparison with children without infection (mean value – 105.3 ± 73.1/mm²) and controls (mean value – 86.1 ± 69.8/mm²). Gastrin cell count in the antrum was higher in moderate infection than in severe infection ($p<0.07$)

The findings on increased G cell counts in inflamed mucosa [12] with no significant difference of G cell counts between patients with and without infection [6] were presented in the

Table 2. **Gastrin levels in serum of children with *H. pylori* infection (I group), after eradication (II group) and in without infection *H. pylori* (III groups), Mann-Whitney U non-parametrical test**

Groups examined	Gastrin levels in serum (μU/ml)								
	n	Min.	Max.	Arith. mean ($\bar{x}$)	Median (M)	Mode	Standard Deviation (SD)	Lower quartile	Upper quartile
Group I	36	59	294	92.9	84.0	62	416	68	99
Group II	15	53	87	63.7	60.3	87	11.3	56	63
Group III	5	70	70	70.0	70.0	-	-	70	70

Figure 2. **ROC curve for gastrin levels in serum of children with *H. pylori* infection**



known literature. Similarly to the study, Kozlowski et al. examining 40 children aged 5-17 years with *H. pylori* infection proved higher G and D cell counts in the antrum and a proportionally higher relation of G/D cells. The values of these parameters decreased after bacterium eradication [12]. However, Rindi et al. evaluating children with antral G cell hyperfunction with and without *H. pylori* infection did not confirm the bacterium influence on an increase in G cell counts [6]. Park et al. and Tzaneva et al. assessing G cell counts in adults with gastritis or gastric or duodenal ulcer in the course of *H. pylori* infection also did not discover significant differences in G cell counts between patients with or without infection. Although D cell count was lower in the infection, the relation of G/D was higher [5,7].

In our study, higher G cell counts in the antrum were found in children with *H. pylori* infection, though no correlation was revealed between G cell counts and the severity of inflammation. In the study, gastrin levels in the serum were significantly higher in group I with gastritis and *H. pylori* infection than in group II without infection ($p<0.01$). Other authors also observed higher gastrin levels in children with *H. pylori* infection [9,13,14,15].

Queiroz et al. examining children with active gastritis in the course of *H. pylori* infection found higher gastrin levels in the serum with accompanying lower somatostatin levels in the antrum when compared to controls [9].

Haruma et al. evaluating gastrin levels in children with duodenal ulcer and in children with antral gastritis demonstrated enhanced gastrin levels in comparison with healthy children.

Gastrin levels were higher in children with duodenal ulcer than in children with antral gastritis. According to the author, the measurement of gastrin levels in the serum can be a marker of subclinical duodenal ulceration, especially among patients predisposed genetically to digestive tract ulceration [13].

Kim et al. testing 51 children showed significantly higher gastrin levels in the serum of children with *H. pylori* infection in comparison with controls. The highest gastrin levels were found in children with duodenal ulceration, lower in children with gastric ulceration and chronic superficial gastritis, and the lowest in superficial gastritis [14].

In our study, no significant differences were found between gastrin levels with regard to the severity of the inflammatory process. Similarly, Oderda et al. observed no difference between gastrin levels in 44 children with *H. pylori* infection [16]. In contrast to our study, Oderda did not discover any correlation between gastrin levels in the serum and *H. pylori* infection (it was comparable in children with and without infection), but simultaneously she observed a significant decrease in gastrin levels after successful eradication [16].

In the study, a correlation between gastrin levels and the age of the examined with past *H. pylori* infection was observed ($p<0.05$). The levels of serum gastrin were higher in younger children and lower in older ones and they became stable between 58 μU/ml and 60 μU/ml. Mc Callion presented similar results confirming that gastrin levels depended on the age of patients [17]. He assessed gastrin levels in 134 children, aged 4-13 years with IgG antibodies against *H. pylori* and proved that the mean gastrin level was significantly higher in younger children (4-5 years, the gastrin level – 155 ng/l) than in older children (12-13 years, the mean gastrin level – 90 ng/l). Early childhood hypergastrinemia in the course of *H. pylori* infection can be associated with temporary achlorhydria accompanying an acute stage of the infection and stimulating gastrin production.

AUC below ROC curve was determined to evaluate the usability of gastrin levels as a diagnostic parameter ($AUC=0.85\pm0.05$ for gastrin in serum) The value of gastrin concentration at the level of 61.38 μU/ml taken as a criteria guarantees the accuracy of diagnosis. The sensitivity criteria was 94.45% and the specificity criteria was 62.5% at this gastrin level.

Summarising the results of our study, gastrin levels in the serum proved to be higher in children with *H. pylori* infection. G cell counts in the antral mucosa were enhanced in children with *H. pylori* infection as well as in children after eradication and they did not differ significantly in both groups.

Conclusions

1. The *H. pylori* infection plays a significant role in the stimulation of G cells increase and gastrin release in the blood serum in children.

2. The eradication of *H. pylori* infection is probably a main factor in gastric secretion down-regulation during gastritis in children.

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The comparison of Health-Related Quality of Life (HRQL) in patients with GERD, peptic ulcer disease and ulcerative colitis

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Abstract

Purpose: The aim of the study was to compare HRQL in patients with three common gastroenterological chronic conditions: gastroesophageal reflux disease (GERD), peptic ulcer disease (PUD) and ulcerative colitis (UC), as well as to assess the correlation between HRQL scores and the disease activity in patients with ulcerative colitis.

Material and methods: The study group comprised of 45 patients with GERD, 35 – with PUD and 30 – with UC. Among patients with UC, 7 were in remission, 13 – in mild active and 10 – with severe phase of the disease, according to Rachmilewitz. HRQL was assessed using 8 domains of Polish version of 36-Item Short Form Survey (SF-36).

Results: The highest mean HRQL scores in all groups were obtained in physical and social functioning SF-36 domains. Among patients with GERD and PUD the worst HRQL results were noted in bodily pain subscale; in patients with UC – in general health perception subscale.

UC patients with remission showed significantly higher HRQL scores compared with those with mild active and severe phase of the disease; especially in social functioning, mental health and vitality ($p<0.001$). Patients with severe UC clinical course had mean HRQL scores statistically lower than those with mild active disease only in vitality and social functioning domains. Mean SF-36 bodily pain parameters were significantly lower in GERD and PUD compared with UC.

Conclusions: All the evaluated diseases have a significant negative impact on patients' HRQL parameters, which needs to be considered in those diseases management. The severity of UC clinical course contributes to impaired HRQL.

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Key words: Health-Related Quality of Life, GERD, peptic ulcer disease, ulcerative colitis.

Introduction

The primary goal of treatment for patients with chronic conditions is to maximize their function in everyday life and to achieve the highest level of well-being [1]. Measures of a disease activity and duration, that are based on the laboratory or endoscopic variables, do not always correlate with well-being, particularly in gastroesophageal reflux disease (GERD) but also in other chronic gastrointestinal (GI) diseases. The whole picture of a patient includes limitations in the work and social activities, home and married life, coping, stressful events. Physicians need precise measures of these outcomes that are also practical for use in the everyday practice [1-3]. The clinical assessment could be focused on Health-Related Quality of Life (HRQL), which describes the psychological and physical functioning and the subjective experience of a person in relation to their health. The point is that when patient is ill almost all aspects of life become health related [4-6].

GI disorders like GERD, peptic ulcer disease (PUD) and ulcerative colitis (UC) are common in general population. Although these diseases are not connected with high mortality rates, they lead to many psychosocial, emotional and economical consequences. On the other hand many stressful events influence on the clinical course of these diseases (e.g. on the relapse of inflammatory bowel disease) [4-8].

Gastrointestinal disorders have a great influence not only on HRQL of diseased persons, but also on their relatives and families. With the onset in young age, they interfere with a very active period of human life. Having long outcome with debilitating duration and possibility of complications (including cancer), these diseases severely affect all aspects of HRQL. Treatment of these conditions requires long-term medical follow-up, frequent invasive endoscopic examinations and continuous drugs' intake [9,10]. Although HRQL was assessed in many chronic

GI disorders, the differences in HRQL in patients with these diseases were not extensively evaluated yet. In addition, the determinants of HRQL in inflammatory bowel disease are not completely understood.

The aim of the study was to compare the HRQL scores in patients with gastroesophageal reflux disease, peptic ulcer disease and ulcerative colitis as well as to assess whether HRQL is associated with UC severity.

Material and methods

Subjects: A total of 110 subjects, (37 men and 73 women), were evaluated.

Subjects were divided into three groups according to the diagnosis:

– patients with gastroesophageal reflux disease (GERD) (n=45; 14 men and 31 women; mean age 52.5±17.6);

– patients with peptic ulcer disease (PUD) (n=35; 13 men and 22 women; mean age 47.3±14.3);

– patients with ulcerative colitis (UC) (n=30; 10 men and 20 women; mean age 43.5±15.0).

Diagnosis: Well established diagnosis of GERD, PUD and UC was based on clinical, laboratory, endoscopic and histopathological measures. GERD was recognized at endoscopy when erosive changes were present. In cases of NERD (30%) – omeprazole-test were performed. Patients with peptic ulcer disease, confirmed by endoscopy with urease test for *Helicobacter pylori*, were in inactive stage of disease and were treated with HP eradication previously. UC was diagnosed at colonoscopy and was confirmed by biopsy with the histopathological examination.

Disease activity: Disease activity in GERD patients were classified with Los Angeles criteria. 20 patients (44%) presented grade A pro LA; 13 (29%) – B pro LA and 6 patients (13%) – C and D pro LA. All patients with PUD have finished treatment and no active disease was revealed in the moment of the study. Disease activity in group of UC patients was assessed by Rachmilewitz' Clinical Activity Index – CAI [11,12]. Remission was considered when the score was maximum 2 points; mild disease 3-8 points and severe disease 9-14 points. Seven of UC patients (23%) were in remission state, 13 (43%) – in mild disease and 10 (34%) – on severe phase of disease.

Treatment: All GERD and PUD patients were treated with antisecretory drugs: proton pump inhibitors (69% and 74%, respectively) or H2R antagonists (33% and 17%). *H. pylori* eradication was performed in 70% of PUD patients. UC patients were treated with sulphasalazine in 17%, sulphasalazine plus steroids – in 27%, mesalazine in 30%, mesalazine plus steroids – in 20% cases.

The demographic data collected were gender, age, marital status, years of education and type of the employment. The medical history included medication, disease duration and severity as well as its extent were taken. Presence of coexisting medical problems and disease complications were noted.

Control group consisted of 40 healthy volunteers (22 men and 18 women; mean age 35.9±9.6).

Informed consent was obtained from all subjects evaluated and the study was approved by local ethics committee.

HRQL assessment

Health-Related Quality of Life was assessed using The Short Form 36 (SF-36) Questionnaire.

The SF-36 is a self-administered generic HRQL measure derived from the Medical Outcomes Study. This questionnaire consists of 36 items covering eight health status domains: physical functioning (PF), role limitation attributable to physical problems – role-physical (RP), bodily pain (BP), general health perception (GH), vitality (VT), social functioning (SF), mental health (MF) and role limitation attributable to emotional problems – role-emotional (RE).

Each domain is scored from 0 to 100, with a higher score indicating better HRQL.

SF-36 evaluates three major health attributes: health status, well-being and overall health status. This method is practical for clinicians and patients, being brief, convenient and providing easy comparison between different populations of patients and healthy subjects. It has been thoroughly tested for validity, reproducibility and responsiveness [13-15]. We used the Polish version of SF-36 [16,17].

Statistical methods

Results were assessed using Statistical Package Online by Quality Metric's responsible for SF-36 distribution and data interpretation.

The differences between the means of analysed parameters in different groups of patients were assessed with the Student's t-test. In order to calculate a significant differentiation in many groups, one-way analysis of variance was performed according to F. Snedecor test. The distribution differences were assessed with the use of non-parametric tests i.e. chi-squared test and Fisher's exact test.

Analysis was made using STATISTICA 5.0 software package, serial number no SP125579705G51.

Results

Demographic data of population studied is shown in *Tab. 1*.

Clinical characteristics of patients:

- Among group of patients with GERD the most frequent symptom (n=34, 75% patients) was heartburn. There were also flatulence (n=32, 71%), upper abdominal pain (n=30, 66%), early satiety (n=28, 63%) and chest pain (n=27, 62%). In some cases extraesophageal symptoms – globus, hoarseness, productive cough were observed. Duration of the disease was variable: 31% patients (n=14) presented one-year reflux history, but the most often the disease was lasted more than ten years (n=26, 58%). We observed comorbidity of reflux and hiatal hernia in 33% cases (n=16). Barrett's oesophagus was detected in 2 patients (4%).
- In group of patients with PUD the most prevalent GI symptoms were: dull epigastric pain – occurring in 80% cases (n=28), flatulence (n=24, 68%), heartburn (n=21, 60%), nausea (n=20, 57%) and vomiting (n=15, 43%). Duration of the disease was: in 7 patients (20%) – one year, in 17 (49%) – up to 10 years and in 11 (31%) – more than 20 years. *Helicobacter pylori* infection was detected in 25 (70%) patients. There were

Table 1. **Demographic features of the study population**

	GERD*	PUD**	UC***	Control
Total (n)	45	35	30	40
Gender				
Male	14 (31%)	13 (37%)	10 (33%)	15 (36%)
Female	31 (69%)	22 (63%)	20 (67%)	25 (64%)
Age (yr)	21-75	17-72	18-75	19-73
Mean age ± SD	52.5±17.5	47.3±14.2	43.5±15.0	45.5±16.0
Marital status (%)				
Single	27	37	33	30
Married	53	51	54	51
Widowed	18	6	3	10
Divorced	2	6	10	9
Education (%)				
Grade school	11	11	20	15
Technical	18	20	17	20
High school	55	49	50	50
University	16	20	13	15
Employment (%)				
Employed	33	29	53	35
Unemployed	13	20	7	12
Retired	52	45	33	47
Student	2	6	7	6

* GERD – gastroesophageal reflux disease; ** PUD – peptic ulcer disease; *** UC – ulcerative colitis

2 cases of PUD complications, as gastroduodenal bleeding and pyloric perforation.

Among 30 patients with ulcerative colitis (UC), 7 (23%) were in remission state, 13 (43%) – presented mild disease and 10 (34%) – severe disease, according to Rachmilewitz' Clinical Activity Index. In 43% (n=13) rectum and sigmoid colon was involved. In 30% cases (n=9) macroscopic pathological changes were observed in rectum, sigmoid as well as distal part of descending colon. Pancolitis was found in 27% patients (n=8). There were 12 cases of extraintestinal symptoms of UC: ankylosing spondylitis – in 1, distal arthritis – in 7, iritis and conjunctivitis – in 4 patients.

GERD – HRQL results

Mean SF-36 subscale scores were lower in patients with GERD than in control group with high statistical significance: $p<0.01$; $p<0.001$ (*Fig. 1*). In the GERD group the best mean scores (62.5±27.9) were obtained in physical functioning domain (PF).

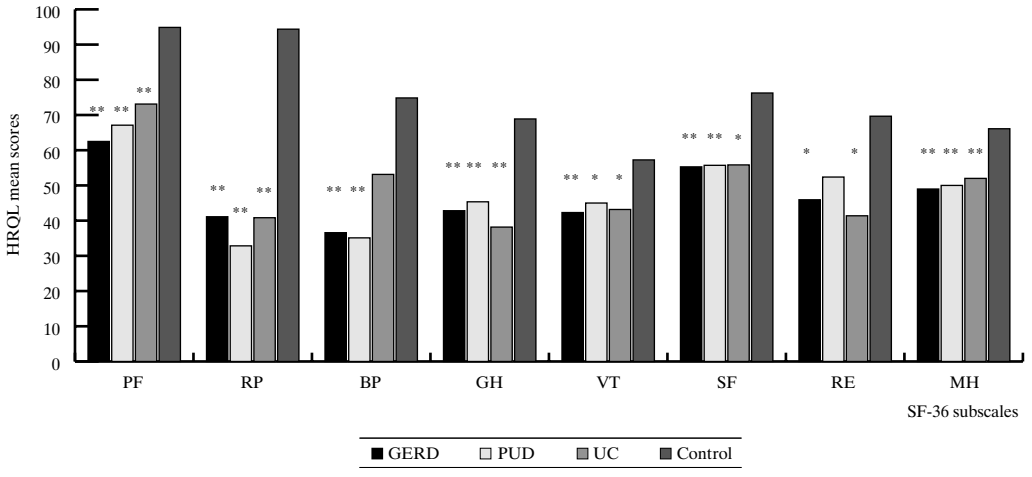
The second best results subscale (55.3±26.8 scores) concerned social functioning (SF). Much worse mean HRQL results were shown in mental health (MH) (48.9±21.3 scores) and role-emotional (RE) subscale (45.4±45.7). Very low and similar mean HRQL scores were presented in vitality (VT) and general health perception (GH): 42.3±19.5 and 42.8±20.1 respectively. Mean score of role-physical (RP) was quite similar (41.1±41.3). The lowest mean scores were observed in bodily pain domain (BP) – 36.6±20.9.

PUD – HRQL results

In almost all SF-36 subscales HRQL of patients with peptic ulcer disease was worse then in control group ($p<0.01$; $p<0.001$). Only within role-emotional (RE) domain the statistical significantly differences were not observed (*Fig. 1*).

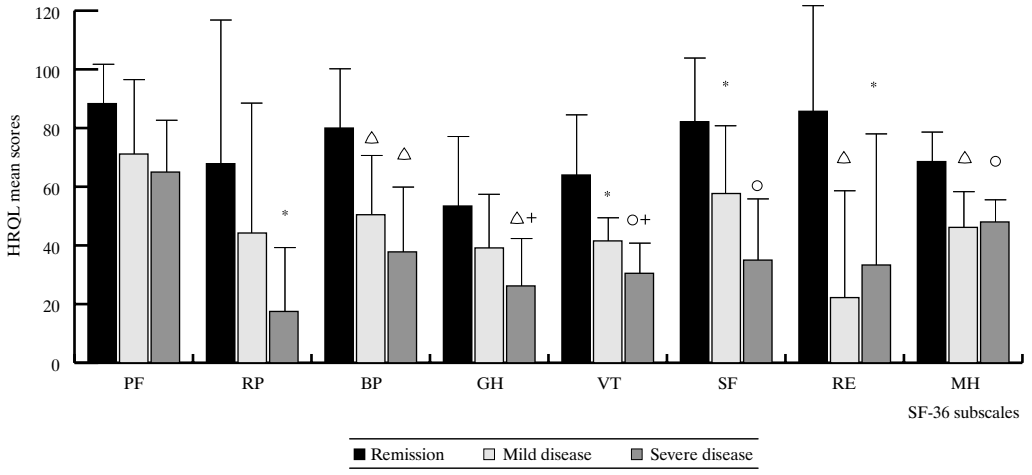
In PUD the best mean scores were obtained in physical functioning subscale (PF) – mean score 67.1±19.7. Remaining SF-36 subscales showed much worse results: social functioning (SF) – 55.7±6.8; role-emotional (RE) – 52.4±45.2 and mental health (MH) – 50.0±18.9. The general health perception, vitality and bodily pain mean scores were even lower: 45.3±22.4; 45.0±21.0 and 35.1±20.9 respectively. The worse HRQL was obtained in the role-physical domain, where mean score was: 32.8±39.9.

Figure 1. **Comparison of HRQL mean scores in patients with gastroesophageal reflux disease (GERD), peptic ucler disease (PUD), ulcerative colitis (UC) and healthy control**



* $p<0.01$ compared to control; **$p<0.001$ compared to control
PF – physical functioning; RP – role-physical; BP – bodily pain; GH – general health perception; VT – vitality; SF – social functioning; RE – role-emotional; MH – mental health

Figure 2. **Correlation of HRQL mean scores and ulcerative colitis (UC) activity**



* p<0.05 compared to remission phase; Δ p<0.01 compared to remission phase; + p<0.05 compared to mild disease; O p<0.001 compared to remission phase

PF – physical functioning; RP – role-physical; BP – bodily pain; GH – general health perception; VT – vitality; SF – social functioning; RE – role-emotional; MH – mental health

UC – HRQL results

HRQL results were lower in the patients with UC than in control group in all domains studied (p<0.01, p<0.001) (*Fig. 1*).

The best HRQL results were obtained in physical functioning domain (PF) – mean score 73.1±21.8. In the remaining subscales, mean scores appeared significantly lower: social functioning (SF) – 55.8±27.6; mental health (MH) – 53.1±25.2; vitality (VT) – 43.2±18.8. Very similar results and low mean scores were observed in the role-emotional and role-physical subscales (41.4±45.1 and 40.8±41.8 respectively). In these group the lowest HRQL was noted in general health perception domain – mean 38.2±20.2.

Furthermore, the correlation between HRQL scores and disease activity was assessed. The best quality of life was observed in patients in remission state.

In patients with the mild UC, the mental health, bodily pain, role emotional (p<0.01), social functioning and vitality (p<0,05) mean scores were significantly lower compared to the patients in remission. However, in other subscales (PF, RP, GH), quality of life in patients with mild disease was not significant different from in those in remission (*Fig. 2*).

A comparison of HRQL parameters of patients in remission and severe phase of the disease showed significantly lower values in the latter in all HRQL domains (p<0.001; p<0.001; p<0.005). Comparing HRQL parameters of patients in mild and severe phase of the disease, these differences were significant only in vitality and general health perception domains (p<0.05).

Surprisingly, in the role-emotional and mental health parameters, higher HRQL scores were seen in severe than in mild disease, however, those differences were not significant. In mental health domain the results of these two groups of patients were very similar – 48.0±8.8 vs 46.1±12.5.

Comparison of the HRQL results in all groups of patients

In all three groups the variables studied in SF-36 domains showed similar results (*Fig. 1*). Only in bodily pain domain in patients with GERD and PUD mean scores were significantly lower then in patients with UC (p<0.002).

Discussion

The generic type of SF-36 questionnaire enabled us to compare the health status of patients with various diagnosis, in different disease activity state and healthy control population. Among subjects studied there were no significant differences in gender and age. We noticed slightly higher percentage of women in all groups, which could be due to their concern about health status and more frequent medical visits. Patients with GERD had significantly lower quality of life scores than control group in all SF-36 domains. Our results confirm the data from previous studies of HRQL in GERD patients [18-21].

Revicki et al. [18] evaluated the association between GERD symptoms and quality of life in 516 patients from US population. Patients with GERD had significantly lower mean scores in all SF-36 subscales compared to healthy subjects. Similarly to our study, the best mean HRQL scores were seen in physical functioning domain. Inversely, the worst results were obtained in vitality, while in Polish GERD patients the worst scores were noted in bodily pain subscale. Perhaps, this is due to the differences in life standards expectations.

Revicki et al. [18] observed higher mean HRQL scores of patients with GERD in physical functioning and role-physical subscales which corresponds with our results. In addition they assessed the effect of 6 weeks GERD treatment with H2R antagonists or proton pomp inhibitors on HRQL. Patients, who responded well to the treatment, had better HRQL scores in almost all SF-36 domains, especially in psychological items.

Results obtained by Leplege et al. [22] in French population were similar to study of Revicki. After 4 weeks of topical anti-inflammatory gel treatment, the treated patients revealed better improvement in all SF-36 domains compared with the placebo group. The HRQL in the treated group reached the level of the French reference population, while it remained impaired in the placebo group. This study gave more data to develop the relevancy of the SF-36 questionnaire to assess the impact of GERD without severe oesophagitis on HRQL.

HRQL assessment is useful particularly in those cases when no pathological changes are seen in traditional medical diagnostic techniques. In small proportion of increasingly recognized non-erosive reflux disease (NERD) no changes in upper GI endoscopy and normal acid exposure can be detected. Watson et al. [23] assessed HRQL of patients with NERD, described as a "sensitive oesophagus" and confirmed their lower quality of life scores compared to healthy population. Similarly to our study, the best results were seen in physical functioning domain, and even before treatment they were better than our PF results (72.9 vs 62.5 scores). Authors revealed significant differences between bodily pain and vitality parameters with omeprazole compared with placebo treatment.

Furthermore, there was no difference in quality of life between patients with GERD, NERD and Barrett's oesophagus in Kulig et al. [24] study. These results confirmed that stages of mucosal damage have little influence on HRQL parameters. These HRQL results were lower than in general population and similar to that of patients after acute coronary events.

In our study HRQL results were also significantly worse in peptic ulcer disease than in control group in almost all SF-36 subscales. Only in role-emotional parameter those differences were not statistically significant.

Rampal et al. [25], used a specific method – Quality of Life in Duodenal Ulcer Patients (QLDUP) questionnaire, which was developed from SF-36 test by addition of 18 questions connected with anxiety, diet, smoking and ulcer pain. Authors showed some differences according to treatment regimen. Apart from therapeutic approach, quality of life reflects therapy acceptance end psychological adaptation to a life-long illness. There is a balance between feeling of therapeutic protection and discomfort due to a prolonged daily intake of drug. Patients could expect that better health is connected with no need for continuous treatment. Even so, maintenance treatment with nizatidine better improved QOL than intermittent therapy. The lowest score was seen in general health perception, what indicate that PUD patients are aware of chronicity of their condition.

Similarly, in our study the worst HRQL scores were obtained in general health perception, and also in the role-physical, bodily pain, and vitality parameters. The best results, however, still lower than in control group, were noted in physical and social functioning. Patient's concerns about the disease chronicity, frequent recurrences, had great impact on quality of life.

Study by Wilhelmsen [26] included 74 patients with PUD treated with triple eradication therapy. It revealed a great improvement in the quality of life after one-year observation. Patients reported better general well-being and emotional state as well as improvement in sexual activity.

Inflammatory bowel disease (ulcerative colitis and Crohn's disease) is highly related to patient's emotional status. Disease relapses are often caused, as considered by patients, by emotional stress [27-29].

In our study the majority of patients were professionally active, developing their careers, therefore the persistent, chronic and recurrent disease did affect their quality of life.

It was not surprising that we noted the significant HRQL impairment in this group of patients compared to the control in all SF-36 parameters. Our results are similar to other studies [10,30,31].

The best scores were obtained in physical and social functioning domains. The general health perception was assessed to be the lowest. There was a significant negative correlation between the HRQL score and the disease severity.

Pallis et al. [30] has also shown that patients with severe disease had significantly lower HRQL scores than patients in remission both in SF-36 and Inflammatory Bowel Disease Questionnaire measures.

Hjorstwang et al. [10] used another generic HRQL test: SIP – Sickness Impact Profile and questionnaire: RFIPC – Rating Form of IBD Patient Concerns. They revealed that the main UC patients concerns were: fear of surgical treatment with ileostomy, side – effects of therapy, incontinence and cancer. These concerns grow with subsequent disease relapses.

Casellas et al. [31,32] measured HRQL in ulcerative colitis using IBDQ. In this study the correlation between less numerous relapses during a year, longer disease duration, better education, male gender, few hospitalizations and better quality of life in IBD patients was shown. In another study by the same author, the negative impact of the disease severity on HRQL using IBDQ and Psychological General Well-Being Index (PGWBI) was confirmed. The best HRQL scores were noticed in social functioning area.

Han et al. [33] showed correlation between elements of physical/mental health measured by the SF-36 and disease-specific quality of life. There was strong relationship between patients' symptoms and all domains of their HRQL, what indicate strategy for improving quality of life in reducing their symptoms.

In our previous study we assessed quality of IBD patients using the self-prepared questionnaire. We have observed the lower HRQL scores in patients with IBD compared to the control group. There was a significant positive correlation between HRQL score loss and the disease severity, particularly in emotional state and perception of physical efficiency. On the other hand in patients with severe disease higher HRQL scores in relations with family and in joy of life domain compared to mild clinical course was observed [34].

The present study provides us with some new data based on Polish population comparing the quality of life in GERD, PUD and UC. Analysis of all mean scores in the diseases studied has shown similar results. However, in bodily pain parameter GERD and peptic ulcer disease patients presented significant lower scores than those with ulcerative colitis. Revicki et al. [18] has also shown the decrease in bodily pain subscale in GERD patients compared to patients with severe depression or diabetes and hypertension. These data further support the thesis that reflux disease severely lowers the pain threshold of the patients.

In the future HRQL evaluations are most likely to be used more frequently, e.g. for more complete assessment of the medical or surgical treatment outcome. It is known that psychological factors, such as HRQL are important predictors of health care utilization. These long-lasting, recurrent diseases, requiring frequent medical consults have a large impact on health care. De Boer et al. [35] underlined that emotional functioning and disease burden experienced are very important in the health care use, therefore psychological interventions may lead to more appropriate allocation of resources and the decrease in costs.

We concluded that quality of life assessment provide a basis for more complete evaluation of the patient and essential supplement for the traditional treatment. The more effective chronic disease management should probably include behavioural and psychological intervention to improve general health state of patients.

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Profiling of peritoneal fluid of women with endometriosis by chemokine protein array

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Abstract

Purpose: Chemokines play essential role not only in controlling leukocyte function and trafficking but also in the angiogenesis and modulation of inflammatory responses.

Material and methods: A novel array-based enzyme-linked immunosorbent assay was used to quantitate peritoneal fluid chemokines of 25 infertile women with endometriosis and 18 controls. For our preliminary studies we chose mini-array containing nine different chemokines: MDC/CCL22, TARC/CCL17, MCP-1/CCL2, RANTES/CCL5, MIP-1α/CCL3, -1β/CCL4, -1δ/CCL15, -3α/CCL20, and -3β/CCL19.

Results: We found significantly higher MIP-3β/CCL19 (P=0.0036) concentrations in peritoneal fluid of women with endometriosis as compared to patients with primary infertility without any signs of disease.

Conclusions: Our preliminary results suggest that MIP-3β/CCL19 might play a role in the pathogenesis of endometriosis but its precise role remains to be established. Novel types of screening methods based on high-throughput technologies offer great opportunities to study immunobiology of endometriosis. It will hopefully provide new possibilities for discovery of new markers and potential drug targets.

Key words: endometriosis, chemokines, peritoneal fluid, protein array.

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Introduction

The pathogenesis of endometriosis, a condition in which the foci of endometrial tissue including epithelial and stromal components are present outside uterus, most often in pelvic cavity, ovaries but also in various distant organs, still remains largely unknown. Since in majority of the cases endometriosis implants are found in the pelvic peritoneum, it was postulated that retrograde menstruation with subsequent implantation of endometrial tissue is a main causative factor [1]. Alternatively metaplasia theory has also been advocated [2] and the evidence of the rare cases of endometriosis in men who are receiving estrogens seems to support it [3].

Regardless of the pathogenesis, which also include other than mentioned-above alternative hypotheses [4], it is evident that in the pelvic peritoneum endometriotic implants are bathed in the peritoneal fluid (PF). A subsequent inflammatory reaction in the pelvis changes the characteristics of the peritoneal fluid and its cellular components [5].

In particular, evidence supports the hypothesis that peritoneal fluid cytokines orchestrate many of the complex processes underlying the initiation and survival of nascent endometriosis implants [6]. Numerous secreted products of the cellular components, such as chemokines, growth factors, matrix metalloproteinases and others have been reported in the PF of women with endometriosis [7-9].

Chemokines (CHEMOtactic cytoKINES), a large multifunctional family of cytokines are structurally related, with most containing four invariant cysteine residues. Depending on the arrangement of the first two of these cysteines, chemokines are divided into four subfamilies: CXC (α), CC (β), C (γ) and CX3C (δ). Chemokines are produced as pro-peptides and are cleaved during secretion to produce an active mature protein that functions by activating G-protein-coupled receptors.

Chemokines have often been co-discovered by multiple investigators and therefore have numerous names in the literature, which can be a source of confusion. A new standardised systemic nomenclature has therefore been adopted [10] and is

used in the article with systemic name following ligands being first mentioned.

It is becoming increasingly evident that chemokines of different types appear to be profoundly involved in the pathogenesis of different diseases of reproductive system, including endometriosis [11]. In our study we intended to perform profiling of nine different chemokines in peritoneal fluid from women with endometriosis by a novel high-throughput mini-array enzyme-linked immunosorbent proteomic assay. Macrophage-Derived Chemokine (MDC, a systemic name – i.e. CCL22), Thymus and Activation-Regulated Chemokine (TARC, i.e. CCL17), Monocyte Chemotactic Protein (MCP-1, i.e. CCL2), Regulated upon Activation, Normally T cell-Expressed, and presumably Secreted (RANTES, i.e. CCL5) Macrophage Inflammatory Proteins (MIP) -1 alpha (i.e. CCL3), -1 beta (i.e. CCL4), -1 delta (i.e. CCL15) -3 alpha (i.e. CCL20), and -3 beta (i.e. CCL19) were chosen for the study. The goal of this study was to demonstrate that the novel array-based high-throughput technologies may offer interesting opportunity to study pathogenesis and to possibly search for new markers of endometriosis.

Material and methods

A total of 42 regularly menstruating women who underwent laparoscopy at Department of Gynaecology of the Medical University in Bialystok, Poland, participated in the study. None of the women had medications that ameliorate endometriosis or modulate the immunological status at least over 3 months prior to laparoscopy. Endometriosis was diagnosed both laparoscopically and histologically. The study was approved by institutional ethics committee and informed consent was obtained from all the patients.

24 patients (group I) were selected by the existence of visible peritoneal endometriotic lesions, characterized as red- or gland-like lesions as well as red vesicles and ovarian endometriomas (stage I-IV) [12]. Of these women, 14 (58.3%) were scored as minimal and mild (I and II stage) of which 11 were those with primary (those who were never pregnant) and 3 with secondary (at least once pregnant previously) infertility. 10 patients (41.7%) were scored as moderate and severe stage (III and IV) of which 5 were primary and 5 secondary infertile women. Eighteen (group II) infertile women (10 with primary and 8 with secondary infertility), without any sign of endometriosis presented at laparoscopy, were used as a control. All laparoscopies were performed in the first phase of menstrual cycle (days 8-12).

Peritoneal fluid samples were collected, cleared of cells and cell debris by means of centrifugation at 800 g for 10 min and then stored at -80°C. We excluded patients with blood-contaminated peritoneal fluid.

A multiplexed, mini-array, sandwich-type, enzyme-linked immunosorbent assay was used to measure in duplicate the concentration of nine different human chemokines: MDC/CCL22, TARC/CCL17, MCP-1/CCL2, RANTES/CCL5, MIP-1α/CCL3, -1β/CCL4, -1δ/CCL15, -3α/CCL20, and -3β/CCL19. The determination (Pierce Biotechnology Laboratory, Rockford, USA) was performed as previously described [13]. The mini-array was produced by spotting monoclonal antibodies (mAbs) in a 3 x 3 pattern in the bottom of the wells of 96-well polystyrene plates. Each antibody captured specific chemokine present in the standards and samples added to the plate. The bound proteins were then detected with a biotinylated detection antibody, followed by the addition of streptavidin-horseradish peroxidase (HRP) and lastly, SuperSignal® ELISA Femto Chemiluminescent substrate. The plate was imaged using SearchLight® Imaging System which is a cooled charge-coupled device (CCD) camera. ArrayVision® customized software was used to quantify the signal from each spot of the array. The amount of luminescent signal produced was proportional to the amount of each protein present in the original standard or sample. Concentrations were extrapolated off a standard curve.

The specificity of the mini-array was demonstrated by adding samples containing only individual chemokines to different wells of a mini-array plate. In each case, the chemokine in the sample produced a signal only at the spot containing the complementary antibody. Low background in the absence of target, and no cross reactivity was observed between the different chemokines and their arrayed antibodies. The dynamic range for the detection of the target chemokines in the array was demonstrated to be: 0.8-800 pg/mL for MCP-1; 3.2-3200 pg/mL for MIP-1α/CCL3 and MIP-1δ/CCL15; 8-800 pg/mL for MIP-1β/ /CCL4; 0.56-560 pg/mL for MIP-3α; 3.2-3200 pg/mL for MIP-1δ; 0.4-400 pg/mL for RANTES/CCL5, TARC/CCL17, MDC/CCL22 and MIP-3β/CCL19.

Total protein in peritoneal fluid was quantified as previously described [14].

As data did not fulfil the normality assumption (assessed by Shapiro-Wilk test) the comparison of studied chemokines was performed by the use of Wilcoxon test. The comparison between subgroups of patients with minimal/mild vs moderate/severe endometriosis vs primary infertile patients without endometriosis was performed using Kruskal-Walis test, whereas Dunn's multiple comparison test was used to show differences between three combination of subgroups i.e minimal/mild primary vs moderate/severe primary, minimal/mild primary vs primary control and moderate/severe primary vs primary control. The comparison between age, BMI as well as duration of infertility was performed by Student t-test. The significance level was equal to 0.05. All tests were two-sided. The statistical analysis was performed by the use of SAS STAT package.

Results

The mean age, BMI as well as duration of infertility of patients in group I and II was similar and did not differ significantly (*Tab. 1*).

The mean volume of peritoneal fluid was 6.1±2.1 ml in study group and 5.0±1.9 ml in controls – a non-significant difference.

When group I and group II were compared by Wilcoxon test no differences were found between women with endometriosis and infertile controls. It was only after division of patients into subgroups, based on the status of infertility (primary vs secondary), when among nine studied chemokines we were able

Table 1. **Clinical characteristics of patients**

	Group I* (mean ±std)	**Group II**** (mean ±std)	**P*****
BMI (body mass index)	23.15±3.17	22.5±4.3	0.48
Age (years)	29.6±4.18	31.8±2.9	0.15
Duration of infertility (years)	4.22±3.56	3.65±1.77	0.64

* – patients with endometriosis (n=24); ** – infertile patients without endometriosis (n=18); *** – p-value of Student t-test for comparison of group I and II

Table 2. **Chemokine levels in primary infertile women with minimal/mild (subgroup I) and moderate/severe (subgroup II) endometriosis as well as primary infertile patients without any signs of disease (subgroup III)**

	Minimal/Mild endometriosis (n=14)*	**Moderate/Severe endometriosis** (n=10)*	**Primary infertility** (n=10)	**P****
MDC	193; **279.4**; 338.1***	268.6; **283.8**; 286.6	213; **237.4**; 269.5	0.25
TARC	16.3; **28.7**; 30.7	17.5; **19.5**; 26.4	21.1; **23.25**; 34.9	0.68
MCP1	704.6; **1289.7**; 2086.5	563.3; **594.9**; 1337.8	566.7; **1178.4**; 1833.2	0.79
RANTES	10.7; **13.8**; 22.4	5.8; **9.6**; 11	11.3; **17.05**; 23.3	0.1
MIP-1α	44; **49.6**; 59.4	50.7; **56**; 66.3	48.4; **51.05**; 61.5	0.58
MIP-1β	24.2; **43.7**; 61.1	23.9; **45.3**; 47.1	33.1; **40.25**; 56	0.82
MIP-1δ	4638.3; **5222.5**; 6296.5	3296.7; **4483.7**; 7021.1	5927.4; **7554.95**;11194.6	0.15
MIP-3α	25.8; **33.6**; 35.6	20.7; **26.9**; 28	27.6; **31.15**; 40.2	0.44
MIP-3β	113.7; **124.5**; 175.8	135.7; **141.8**; 239.9	68.9; **77.05**; 88.55	0.0007#

* – Primary infertile patients; ** – P-value for Kruskal-Walis test; *** – Data in pg/mL: Q1; Median; Q3 (Q1-Q3 are interquartile values); # – Statistically significant differences

to observe significantly (p=0.0014) higher concentrations of MIP-3β/CCL19 [129.4 pg/mL (interquartile values Q1: 118.05 – Q3: 186)] in patients with endometriosis than those with primary infertility [77.05 pg/mL (interquartile values Q1: 68.9 – Q3: 88.55)]. We also found that the differences are significant when a group I (of patients with endometriosis) was subdivided into minimal/mild (subgroup I) and moderate/severe (subgroup II) - both with primary infertility and then compared with primary infertile women (subgroup III) without any signs of endometriosis (see details *Tab. 2*). By the use of Dunn's multiple comparison test we could show that no significant difference exists between subgroup I and II (p>0.05), however, it does exist between subgroup I and III (p<0.01) as well as II and III (p<0.001). There is also no significant difference (P=0.25) in duration of infertility between subgroup I, II and III (mean ±SD: 3.4±2.33; 6±5.3; 3.65±1.77, respectively for three subgroups).

The concentration of total protein in peritoneal fluid was not different significantly and it was respectively 4.9±0.89 g/dL and 5.3±0.43 g/dL in the study and control groups.

We did not find any statistically significant differences between secondary infertile patients with minimal/mild and moderate/severe endometriosis as compared to secondary infertile women without any signs of disease.

Discussion

The genomics and proteomics revolution has introduced a paradigm-shift in the experimental approach, which tends to generate, rather than test, hypotheses. This has become possible by the widespread application of high-throughput technologies such as gene and protein microarrays. The concept of using different types of arrays has been revolutionising biomedicine and its application to proteomics is becoming increasingly popular. Majorities of the hitherto applied protein array systems have, however, demonstrated the ability to detect qualitative interactions between proteins. Only few exhibited the real ability of multiplexed quantification of proteins [15,16]. An interesting option has recently been demonstrated by Moody et al. who applied enzyme-linked immunosorbent assay for the qualitative measurement of different cytokines from a single sample [13].

In our study we used exactly the same method for determination of nine different chemokines in peritoneal fluid obtained from women with endometriosis. The choice of the profiled chemokines was driven not only by the fact that their role in the immunobiology of endometriosis is practically unknown but was also prompted by accumulating evidence for a significant contribution of many different chemotactic cytokines in the pathogenesis of endometriosis [11,17].

In the present study we found higher MIP-3β/CCL19 concentrations in peritoneal fluid of women with endometriosis, regardless of the severity, as compared to patients with primary infertility without any signs of disease. The chemokine MIP-3β/CCL19 has not to our knowledge been previously found to be connected with pathophysiology of endometriosis and the role of MIP-3β/CCL19 in relation to affected women is speculative.

Chemokines, in general, affect a number of different tissues and organs not only by the endocrine mode of action through influencing distant organs but also acting locally, possible also in the peritoneal fluid. MIP-3β/CCL19 is a chemoattractant for different types of cells, including natural killer cells [18] as well as T and B lymphocytes [19].

In the reproductive system, MIP-3β/CCL19 has recently been found to be expressed in endometrium [20], and its lower concentrations we found in serum of women with preterm labor [21]. However the precise role of MIP-3β/CCL19 as a chemoattractant of endometrial natural killer cells or as a specific immunological mediator in endometriosis remains unclear.

It is also speculative what might be the source of MIP-3β/ /CCL19 in the peritoneal fluid. Cells in the PF are mainly mononuclear cells, particularly macrophages (90%); other mononuclear cells that are present include lymphocytes and natural killer [22] cells. It has been generally accepted that in peritoneal fluid of women with endometriosis, the number and activity of macrophages and its cytokines is increased. On the contrary, NK cell number and activity has been found to be decreased in peripheral blood and peritoneal fluid of women with endometriosis [23,24]. It would obviously be of interest to correlate the results obtained for MIP-3β/CCL19 with different cellular subtypes and this is now being contemplated as part of the future studies.

The results of our study might seem somewhat surprising given the fact that two other chemokines i.e: RANTES/CCL5 and MCP-1/CCL2 were previously found to be increased in peritoneal fluid of women with endometriosis [25-27]. This, however, may be owing to at least three inherent drawbacks of our study. First concerns lower number of patients recruited as compared to studies cited above. Most of the patients in the moderate/severe group were, however, diagnosed with endometrial cysts and abundant adhesions with lesser visible pelvic lesions. It is well accepted that biological activity of endometriosis tends to weaken in the most advanced cases as described above (IV stage) and immunological milieu of the peritoneal cavity is somewhat different from the III and lower stages thus contributing to lowering of normally increased concentrations of some of the chemokines, possibly RANTES/CCL5 and MCP-1/CCL2.

It was previously found that mean RANTES/CCL5 concentrations in the PF of patients with mild endometriosis are particularly high i.e. 7.8 ng/mL for mild and 29.1 ng/mL for the severe group [28]. In the more recent study Bersinger et al. found significantly lower median PF RANTES/CCL5 levels of 22.5 pg/mL in the mild and 35.5 pg/mL in the severe group of patients [29] whereas in our study the median values were even lower i.e 13.8 pg/mL and 9.6 pg/mL, respectively. The same might apply to MCP-1/CCL2 which was previously found to be increased in women with endometriosis and the median peritoneal fluid MCP-1/CCL2 level was 144 pg/ml (range 54--261) in endometriotic women without adhesions and 336 pg/ml (range 130-2494) in women with adhesions [28]. The ranges and median values of MCP-1/CCL2 were in our studies significantly higher (see *Tab. 2*). Whether the difference in the above studies and ours are owing to the size of study group or might also result from different methodologies (commonly used classical ELISA vs novel array-based ELISA system) remains to be seen in the future expanded studies on larger group of patients who are now ongoing in our department.

Another drawback may also concern both the structure of control group, which is limited only to infertile patients, and the collection of studied patients which was restricted to the follicular phase of the cycle. It is therefore difficult to evaluate whether control group is a healthy one alone since infertility might to some degree influence different chemokine levels. Nevertheless, in Poland it is a difficult task to collect patients who undergo diagnostic laparoscopic procedure for other reasons than infertility and/or pelvic pain and the operations in our Department are conducted only in the first phase of the cycle. We are also now in the process of collecting samples from patients with pelvic pain to see whether any differences might exist between two groups of patients.

Regardless of the explanation as to the potential role of MIP-3β/CCL19 we believe that in general the above-presented preliminary findings create a basis for future confirmatory studies that would hopefully add another brick to the ever-rising wall of the knowledge about immunobiology of endometriosis. This is becoming a possible thanks to the use of a novel multiplexed proteomic assay. By relatively small amount of examined factors, nine altogether, we were able to start laying groundwork for one more hypothesis about the role of chemokines in the pathogenesis of endometriosis.

Acknowledgements

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Peptic ulcers and oral health status

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Abstract

Purpose: *Helicobacter pylori* infection plays a crucial role in pathogenesis of peptic ulcers; however, among infected individuals only a small percentage will develop peptic ulcers at any time during their life. This low virulence suggests that many additional factors beside *H. pylori* are implicated in pathogenesis of the disease. The aim of the study was to determine whether there is a relationship between the prevalence of peptic ulcers and oral health status.

Material and methods: The evaluation of dental status was performed in *H. pylori* infected population. The study involved 93 peptic ulcer patients (77 duodenal ulcer, 16 gastric ulcer) with ulcer niche not related to non-steroidal anti-inflammatory drugs (NSAIDs) consumption and 93 gender and age matched dyspeptic controls. *H. pylori* infection was determined in endoscopically taken slices from gastric mucosa with two methods (CLO-test and histology).

Results: Both in duodenal and gastric ulcer patients, the number of filled teeth was lower and debris index was higher than in controls, the number of decayed teeth was also higher in gastric ulcer patients. The number of natural teeth, number and type of prosthetic restorations, as well as the periodontal index, did not differ between the ulcer and control groups. Poor oral health in patients with peptic ulcers corresponded with education level, smoking habit, and visits to the dentist.

Conclusions: Poor oral health is associated with the prevalence of peptic ulcers not related to NSAIDs consumption, but it appears doubtful that it is a significant pathogenetic factor in ulcer disease.

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Introduction

Half of the world population is infected with *H. pylori*; however, a strong pathogenetic association of *H. pylori* infection was only found with peptic ulcer disease. Since only a small part of the infected population develops peptic ulcers [1], the presence of *H. pylori* infection alone is inadequate as the sole etiological agent to duodenal and gastric ulcer development. This means that in the pathogenesis of this disease, additional factors must be involved.

Former studies indicated that *H. pylori* can colonize not only the stomach, but also the oral cavity; a strain has been isolated from dental plaque and its implication in stomach infection has been proven [2-4]. Poor oral health predisposes patients to more frequent infection of the stomach and makes elimination more difficult during eradication therapy [5-7]. Whether other factors than oral infection with *H. pylori* implicate the oral cavity to peptic ulcer development remains to be established. Although significant consideration has been given to the link between oral health and peptic ulcer disease, this association has been suggested but never confirmed. Therefore, the aim of the study was to determine whether there is association between the prevalence of peptic ulcers and oral health status in the *H. pylori*-infected population.

Material and methods

The study was performed in 186 *H. pylori* infected subjects. Among them were 77 duodenal ulcer and 16 gastric ulcer patients with ulcer niche not related to consumption of non-steroidal anti-inflammatory drugs (NSAIDs) and 93 gender and age matched dyspeptic controls without ulcer history and normal upper GI endoscopy (*Tab. 1* and *2*). All participants

Table 1. **Data of non-NSAIDs-related duodenal ulcer patients and controls**

	Duodenal ulcer	Control	
N	77	77	
Age	48.0±14.5	48.2±14.2	
Gender (M/F)	58/19	58/19	
Education (E,J/ S,U)	43/34	31/46	
Smokers	49	32	p=0.0096
Dentist visits			
(regular/ non-regular)	11/66	31/46	p=0.0005
Number of teeth	16.6±10.0	18.8±8.7	
Number of decayed teeth	4.4±4.0	3.1±3.0	
Number of filled teeth	3.2±4.0	5.7±4.2	p=0.0000
Plaque index	1.5±0.8	1.1±0.8	p=0.0076
Periodontal index	1.7±2.0	1.2±1.3	
Denture wearers	36	44	
Fixed	7	15	
Removable	22	19	
Fixed and removable	7	10	
Total time of daily toothbrushing			
0 min	23	14	
less than 2 min	12	12	
2 min or more	37	47	

Education level consists of elementary school (E), junior high school (J), senior high school (S), and university (U)

Table 2. **Data of non-NSAIDs-related gastric ulcer patients and controls**

	Gastric ulcer	Control	
N	16	16	
Age	57.8±14.1	57.9±13.8	
Gender (M/F)	8/8	8/8	
Education (E,J/ S,U)	11/5	6/10	
Smokers	9	4	
Dentist visits			
(regular/ non-regular)	1/15	5/11	
Number of teeth	12.5±9.3	13.7±9.4	
Number of decayed teeth	6.2±4.8	2.0±1.4	p=0.027
Number of filled teeth	1.3±4.8	4.9±4.4	p=0.032
Plaque index	1.9±0.9	0.8±0.7	p=0.003
Periodontal index	2.5±2.4	1.4±2.0	
Denture wearers	6/16	9/16	
Fixed	1	3	
Removable	4	6	
Fixed and removable	1	0	
Total time of daily toothbrushing			
0 min	5	4	
less than 2 min	3	0	
2 min or more	5	10	

underwent endoscopy with GIF V2 gastroscope (Olympus) during which slices of gastric mucosa from prepyloric and corpus regions were taken; two slices from each side for histological examination, one for CLO-test. *Helicobacter pylori* infection in the stomach was determined using two methods, rapid urease test (CLO-test) and histological examination. Urease test was prepared in the Department of Physiology Medical University of Białystok according to the method of Marshall et al. [8]. The result of the test was considered positive if during incubation at room temperature it changed color from orange to pink; positive and negative controls were performed each day. Specimens for histological examination were placed in formalin, submitted to standard processing, and then stained with hematoxylin-eosin and Giemsa. One experienced pathomorphologist made microscopic assessment. Only subjects with two positive tests for *H. pylori* were included.

Demographic data, oral hygiene practices, and dentist visits were based on questionnaire fulfilled by all subjects before oral examination. The dentist visits were classified as regular if taken at least once a year.

Oral examination involved the evaluation of natural dentition (number of natural teeth, carious teeth, filled teeth, plaque index, periodontal index) [9,10], and specification of dental prosthesis (fixed, removable).

The results (means ±S.D.) were evaluated statistically with Mann-Whitney U-test, χ^2 test, and Fisher exact test, as appropriate. A p value of less than 0.05 was accepted as statistically significant.

The study was approved by the Local Ethical Committee and informed consent was obtained from all participants before study began.

Results

Both in the gastric and duodenal ulcer patients the number of filled teeth was lower and debris index was higher than in controls (*Tab. 1*); additionally, in gastric ulcer patients, the number of decayed teeth was higher (*Tab. 2*). If the two ulcer groups were pooled, they exhibited lower educational level and less regular visits at the dentist than pooled control groups (*Tab. 3*). The number of natural teeth, number and type of prosthetic restorations, periodontal index, as well as total time of daily toothbrushing did not differ between compared groups. Moreover, most peptic ulcer patients, unlike controls, were smokers.

Discussion

The infection of the stomach with *H. pylori* appears to be the most important factor in pathogenesis of peptic ulcer disease. However, many factors in addition to *H. pylori* infection predispose patients to peptic ulcer development, among them are family history, male gender, strenuous work, tobacco smoking, low educational level and low socio-economic status [11-15]. Since stomach infection with *H. pylori* is the main, but not the only, cause of peptic ulcer development, we have attempted to evaluate the role of other potentially important pathogenetic factors of this disease. The aim of the study was to answer the question whether there is association between the prevalence of peptic ulcers and oral health. Assuming that peptic ulcers located both in the stomach and duodenum may sometimes be a side effect of NSAIDs, the present study has been designed to include only patients with peptic ulcers unre-

***Table 3*. Data of non-NSAIDs-related peptic ulcer patients and controls**

	Peptic ulcer	Control	
N	93	93	
Age	49.6±14.8	49.9±14.5	
Gender (M/F)	66/27	66/27	
Education (E,J/ S,U)	54/39	37/56	p=0.018
Smokers	59	36	p=0.013
Dentist visits (regular/ non-regular)	12/81	36/57	p=0.0001
Number of teeth	15.9±9.4	17.9±9.0	
Number of decayed teeth	4.7±4.2	2.9±2.8	p=0.0066
Number of filled teeth	2.9±3.8	5.6±4.2	p=0.0000
Plaque index	1.5±0.8	1.1±0.8	p=0.0003
Periodontal index	1.8±2.1	1.2±1.4	
Denture wearers	42	53	
Fixed	8	18	
Removable	26	25	
Fixed and removable	8	10	
Total time of daily toothbrushing			
0 min	29	18	
less than 2 min	15	12	
2 min or more	42	57	

lated to NSAIDs consumption. Moreover, since dental status depends upon gender and age, these factors were taken into account in recruitment.

The results of this study have shown that oral health status in *H. pylori* infected subjects with peptic ulcer unrelated to NSAIDs is worse than in controls, and a number of oral health factors could be the cause of this effect. Poor dental status in patients with peptic ulcer unrelated to NSAIDs may be the consequence of less care of oral health. Rare visits to the dentist and short time of daily brushing natural teeth may lead to dental plaque accumulation, and finally to dental decay responsible for early tooth loss. It is of note that patients with gastric and duodenal ulcers were of lower education level than controls and thus quite likely of lower economic status; these both may limit markedly the means and possibility of keeping up standards of oral health care. Moreover, most patients with peptic ulcers were smokers, and this factor may have additional contribution to poor oral heath [20].

Poor dental status may be a consequence of a restricted diet. It is possible that the dietary habits of the ulcer population were different from the general population, and shifts in food selection patterns with insufficient intakes of some nutrients and vitamins might be the cause of dental invalidity [16]. An argument for this is an increase of patient body weight within one year after ulcer healing [17].

On the other hand, poor dental status may be considered as a possible cause of peptic ulcers. Less effective mastication may lead to swallow of food particles of relatively large size which reside in the stomach much longer than well masticated [18,19]; a delayed stomach emptying predisposes to gastritis, and finally to disturbed gastric acid secretion, a crucial pathogenetic factor in peptic ulcer development. The unanswered question remains whether impaired mastication could promote gastric and duodenal ulcer in the same way, as it is known that pathogenesis of these two entities differs at many points.

The limitation of the study is that the control group consisted of dyspeptic patients but not of healthy subjects; however, only this model was possible to be accepted from ethical point of view. Despite limitations, the obtained results confirm the suggestion that poor oral health is associated with the prevalence of peptic ulcers unrelated to NSAIDs, but this association in *H. pylori* infected population is rather incidental.

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Antimitotic activity of high affinity ligands for peripheral benzodiazepine receptor (PBR) in some normal and neoplastic cell lines

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Abstract

Purpose: Overexpression of PBR has been found in several tumor types including ovarian, colon, breast adenocarcinomas, esophageal cancer. There is evidence suggesting that PBR ligands regulate cell proliferation. However, their action is probably cell-type specific. We decided to evaluate mitotic activity of PBR ligands in some normal and neoplastic cell lines.

Material and methods: The cells were maintained according to standard procedures. Ligand binding assay was performed in cell extract using PK-11195 or Ro-54864 and [N-methyl-^{3}H] Ro-54864 or [N-methyl-^{3}H] PK-11195. Cell proliferation was evaluated using 5-[^{3}H]-thymidine assay. Western Immunoblot assay was conducted using polyclonal anti-PBR antibody.

Results: We have found that, macrophages evoked strong binding of both Ro-54864 and PK-11195. This phenomenon was accompanied by drastic decrease in the cell divisions. Similar effect was found only in the case of non-estrogen-dependent breast cancer cells MDA-MB 231. It suggest that PBR-ligand mediated inhibition of mitogenesis may represent a new anticancer strategy in non-estrogen-dependent breast cancer. In respect to macrophages inhibition of the cell division by both PBR ligands may have implication in modulation of inflammatory response. It has been postulated that PBR ligands may have anti-inflammatory activity in rheumatoid arthritis. The presence of peripheral benzodiazepine receptors in chondrocytes, T cells, macrophages and mesenchymal cells suggest that peripheral benzodiazepine receptor ligands may interfere with the cytokine network and thus modulate inflammatory response.

Conclusions: The data suggest that PBR-ligand mediated inhibition of DNA synthesis in non-estrogen dependent breast cancer cells and in macrophages may represent a new therapeutic approach of breast anticancer and anti-inflammatory therapy.

Key words: peripheral benzodiazepine receptors, Ro-54864, PK-11195, macrophages, neoplastic cell lines.

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Introduction

The peripheral – type benzodiazepine receptor (PBR) is a multimeric complex composed of 18 kDa receptor protein, the 32 kDa voltage anion-dependent channel (VDAC) protein required for benzodiazepine binding [1] and 30 kDa adenine nucleotide carrier [2]. PBR is present in most tissues. It is particularly abundant in steroid producing tissues [3], it is found in the outer mitochondrial membrane [4] and the outer – inner mitochondrial membrane contact sites [5]. Using high affinity PBR drug ligands such as the isoquinoline carboxamide PK-11195, it was shown that PBR is involved in the transport of the substrate cholesterol into mitochondria [6], the rate – determining step in steroid biosynthesis. PBR has been shown to be implicated in mitochondrial respiration [7], apoptosis [8] and cell proliferation [9]. Overexpression of PBR attenuates apoptosis induced by reactive oxygen radicals or ultraviolet light [10]. Overexpression of PBR has been found in several tumor types including ovarian, colon, breast adenocarcinomas [11], esophageal cancer [12].

The benzodiazapine Ro-54864 and isoquinoline carboxamide PK-11195 exhibit nanomolar affinity for PBR. PK-11195 has been classified as an antagonist and Ro-54864 as an agonist of the receptor [13]. Isoquinoline carboxamide bind specifically to the 18 kDa subunit, whereas benzodiazepine ligands such as Ro-54864 bind to a site consisting of porin, as well as adenine nucleotide translocase and 18 kDa subunit [1].

Several lines of evidence suggest that PBR ligands regulate cell proliferation [14]. However, their action is probably cell-

type specific. Therefore, we decided to evaluate mitotic activity of PBR ligands in some normal and neoplastic cell lines.

Materials and methods

Materials

1-(2-chlorophenyl)-N-methyl-n-(1-methylpropyl)-3-isoquinoline carboxamide (PK-11195), 4'-chlorodiazepam (Ro--54864), BCIP/NBT reagent as well as other reagents were from Sigma-Aldrich (St. Louis, MO). [N-methyl-^{3}H]-Ro-54864 (74 Ci/mmol), [N-methyl-^{3}H]-PK-11195 (83,5 Ci/mmol) – PerkinElmer (Boston, MA). [^{3}H]-thymidine (6,7 Ci/mmol) – ICN. D-MEM with Glutamax I, D-MEM, L-15 Leibovitz, Fetal Bovine Serum (FBS), Penicillin, Streptomycin, phosphate buffered saline (PBS) – Gibco (USA). Glas fiber filters were from Whatman (USA). Anti-PBR antibody – Laboratory of Cellular and Molecular Interactions, University of Rennes (France).

Cell culture

Normal human skin fibroblasts, macrophages, estrogen-dependent breast cancer cells MCF-7 and endometrium cancer cells – Ishikawa were maintained in D-MEM with GlutaMax I supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. Non estrogen-dependent breast cancer cells MDA-MB 231 cells were maintained in D-MEM with 2% glutamine supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C. Fibroblasts were used in the 8th to 14th passages.

Western immunoblot

Samples of cell extracts (25 μg of protein) were electrophoresed. The proteins were transfered to 0.2 μm pore-sized nitrocellulose at 25 V for 1 h. The nitrocellulose was incubated with polyclonal antibody against PBR at concentration 1:500 overnight. In order to analyze PBR alkaline phosphatase conjugated antibody against rabbit's Fc IgG was added at concentration 1:3000 in TBS-T. Incubation was continued for 1 h. Then nitrocellulose was washed with TBS-T (5 times for 5 minutes) and submitted to Sigma-Fast BCIP/NBT reagent.

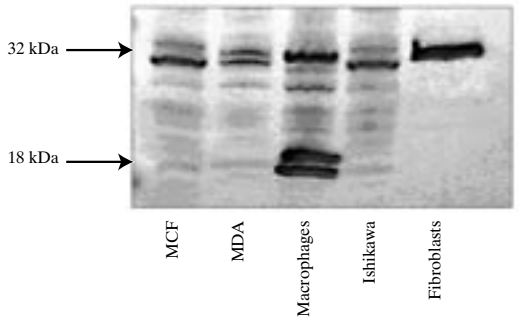
Ligand binding assay

Cell extracts (80 μg) were incubated with [N-methyl-^{3}H]Ro--54864 (30000 cpm) or [N-methyl-^{3}H]PK-11195 (30000 cpm) with or without Ro-54864 or PK-11195 for 3 h at 4°C. Incubation was terminated by transfer of the samples to PBS-prewetted filters and immediate vacuum filtration. The filters were washed three times with PBS and punched into vials and radioactivity was measured. Specific binding was calculated as a difference between total and non-specific binding.

[^{3}H]-thymidine incorporation

To examine the effect of Ro-54864 and PK-11195 on the cells proliferation, the cell were seeded in 24 well plates at 1 x 10^5 cells/well with 1 ml of growth medium. After 48 hours to subconfluent cells various concentrations of the ligands and 0.5 μCi of 5-[^{3}H]-thymidine were added. The incubation was continued for 24 hours at 37°C. Cells were rinsed 3 times with PBS, solubilized with 1 ml of 0.1 mol/l sodium hydroxide containing 1% SDS, then scintillation fluid "Ultima Gold XR" was added and incorporation of the tracer into DNA was measured in scintillation counter.

Figure 1. **Western immunoblot analysis for PBR in different neoplastic (MCF-7, MDA-MB 231, Ishikawa) and normal (human dermal fibroblasts, macrophages) cell lines. The studied cells were cultured in the same conditions to 80% of confluency in D-MEM supplemented with 10% FBS. Samples used for electrophoresis consisted of 25 μg of protein of cell extracts**



Statistical analysis

In all experiments, the mean values from six assays ±standard deviations (SD) were calculated. The results were submitted to the statistical analysis using the Student's t-test, accepting $p<0.05$, as significant.

Results

The expression of peripheral benzodiazepine receptor (PBR) was studied in breast cancer cell lines; MCF-7 (estrogen-dependent) and MDA-MB 231 (non-estrogen-dependent) cells, in cancer cell line from endometrium (Ishikawa) as well in some normal cell lines; human dermal fibroblasts and human macrophages. All studied cells expressed PBR of molecular weight about 32 kDa, while macrophages expressed in addition 18 kDa receptor (*Fig. 1*). The expression of 32 kDa PBR, however, was mostly pronounced in fibroblasts and macrophages.

The differences in expression of PBR among studied cells may suggest their different ligand binding capacity. One of the high affinity ligands for PBR is Ro-54864 [13]. As shown on *Fig. 2* the most binding of Ro-54864 was found in homogenate extracts of macrophages and Ishihawa cells. Much less binding was observed in breast cancer cells and very little binding in human fibroblasts.

Another high affinity ligand for PBR is PK-11195, that may evoke inhibition of biological activity of the receptor [13]. However, in this case binding of PK-11195 to cell homogenate extracts was found to be very weak in both breast cancer cell lines and fibroblasts and much lower in macrophages and Ishikawa cells, compared to the results of Ro-54864 binding in these cells (*Fig. 3*).

The differences in binding of both ligands to PBR suggest, that this phenomenon may affect DNA biosynthesis and subsequently mitotic divisions in studied cells. Therefore, [^{3}H]-thymidine incorporation assay was performed in studied

Figure 2. **Binding of Ro-54864 in cell extracts of various cell lines**

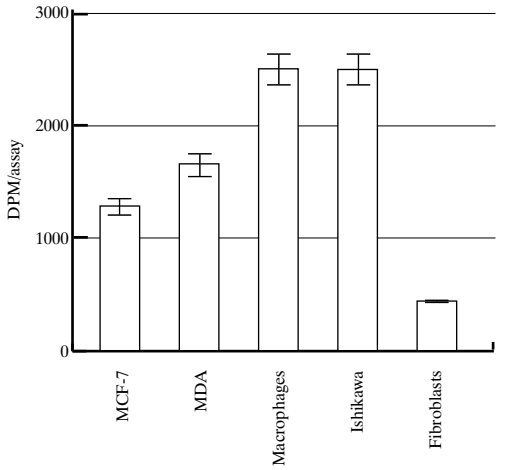


Figure 3. **Binding of PK-11195 in cell extracts of various cell lines**

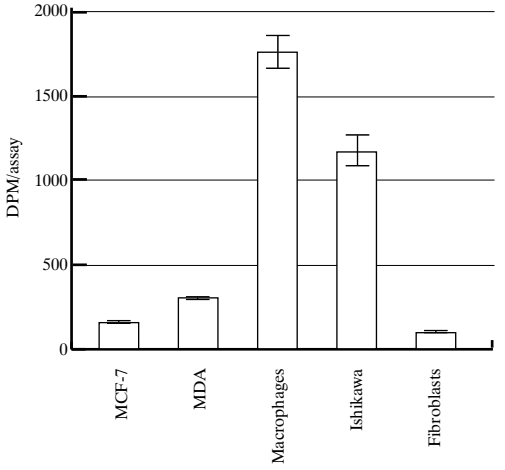


Figure 4. **DNA biosynthesis (measured by 5-[^{3}H]-thymidine incorporation assay) in subconfluent cells treated with different concentrations of Ro-54864 (30, 100 and 300 mM) for 24h in DMEM supplemented with 10% FBS. Mean values from three independent experiments done in duplicates are presented**

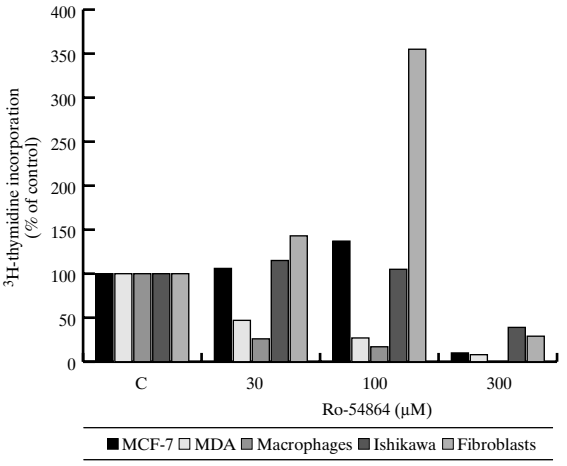
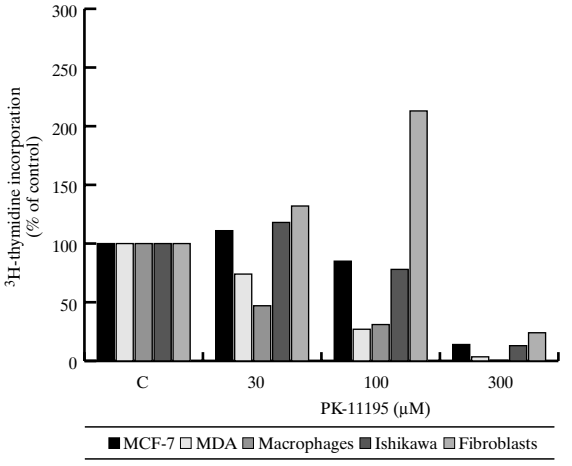


Figure 5. **DNA biosynthesis (measured by 5-[^{3}H]-thymidine incorporation assay) in subconfluent cells treated with different concentrations of PK-11195 (30, 100 and 300 mM) for 24 h in DMEM supplemented with 10% FBS. Mean values from three independent experiments done in duplicates are presented**



cells treated with different concentrations of Ro-54864 or PK-11195, as described in "Materials and methods" section. In the MDA-MB 231 cells and macrophages treated with 30 μmol/l of Ro-54864 the DNA biosynthesis was significantly decreased, while in MCF-7 cells, Ishikawa and fibroblasts it was not much changed. Increase in the concentration of Ro-54864 to 100 μmol/l augmented the inhibition of DNA synthesis in MDA-MB 231 cells and in macrophages, in MCF-7 the ligand slightly stimulated the process, while in fibroblast it was two fold increased. Exposure of the cells to 300 μmol/l of Ro-54864 was found to be toxic for all studied cells (*Fig. 4*).

In case of PK-11195 similar concentration-dependent effects on DNA synthesis in all studied cells was observed (*Fig. 5*).

Discussion

Although several lines of evidence suggest that PBR ligands can regulate cell proliferation [11,12] their action is controversial. In this report we presented that all studied normal and neoplastic cells express PBR. However, functional significance of PBR expression is studied by using high affinity PBR ligands. In this study we used PBR agonist Ro-54864 and PBR antagonist PK-11195. All studied cells, except fibroblasts evoked intense binding of Ro-54864. Similar level of PK-11195 was found only in Ishikawa cells and macrophages. It suggests cell-type specific PBR binding. In fact it is known that PBR function is particularly important in steroid producing cells [3]. However, we provide evidence that other cells that are not involved in steroidogenesis strongly bind PBR.

We have found that, macrophages evoked strong binding of both Ro-54864 and PK11195. This phenomenon was accompanied by drastic decrease in the cell divisions. Similar effect was found only in the case of MDA-MB 231. It suggest that PBR--ligand-mediated inhibition of mitogenesis may represent a new anticancer strategy in non-estrogen dependent breast cancer. In respect to macrophages inhibition of the cell division by both PBR ligands may have implication in modulation of inflamma-

tory response. It has been postulated that PBR ligands may have anti-inflammatory activity in rheumatoid arthritis.

The peripheral benzodiazepine receptor anti-inflammatory effect may also be explained by modifications of cytokines level. Cartilage destruction is a major characteristic of rheumatoid arthritis and is also linked to abberant cytokine and growth factor expression in affected tissues [15]. Interleukin-1, tumor necrosis factor-α and interferon-gamma are known to affect chondrocytes function [16-18], and interleukin-6 has been shown to boost progression from initial inflammation to a chronic state [19]. Peripheral benzodiazepine receptor ligands are known to reduce macrophage secretion of interleukin-1, interleukin-6 and TNF-α [20]. Furthmore, Ro-54864 and PK-11195 dramatically reduce both IL-6 and IL-13 expression in pleural exudation of mice injected with carrageenan [21]. The presence of peripheral benzodiazepine receptors in chondrocytes, T cells, macrophages and mesenchymal cells suggest that peripheral benzodiazepine receptor ligands may interfere with the cytokine network and thus modulate inflammatory response.

Those data suggest that PBR-ligand mediated inhibition of DNA synthesis in non-estrogen dependent breast cancer cells and in macrophages may represent a new therapeutic approach of anti-inflammatory and breast anticancer therapy.

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Myeloid and lymphoid dendritic cells and cytotoxic T lymphocytes in peripheral blood of non-small cell lung cancer patients – a pilot study

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Abstract

Purpose: Dendritic cells (DCs) and cytotoxic T lymphocytes (CTLs) are the first protecting barrier against different pathogens (viruses, bacteria and neoplasms cells). Immature myeloid- and lymphoid dendritic cells possess ability to phagocytose and present antigens to lymphocytes. They have also ability to produce IL-12, which is also known as natural killer cell stimulatory factor or cytototoxic lymphocyte maturation factor. The aim of the study was to demonstrate the relationship between percentage of immature dendritic cells and percentage of CTLs subtypes in peripheral blood of non-small cell lung cancer (NSCLC).

Material and methods: The study population consisted of 10 patients suffered from NSCLC (the mean age: 61.8±10.55). The monoclonal antibodies and three-color flow cytometry technique was applied to determine the cells phenotype in peripheral blood.

Results: Significant negative correlation ($R=-0.693$, $p<0.05$) between percentage of lymphoid DCs and percentage of CTLs was shown. The myeloid to lymphoid DCs ratio significantly positively ($R=+0.638$, $p<0.05$) correlated with the percentage of CTLs. The significant negative correlation between the percentage of myeloid DCs and the percentage of CTLs-IL-12R-positive cells, as well as expression of this receptor were also ascertained ($R=-0.68$, $p<0.05$ and $R=-0.757$, $p<0.01$, respectively).

Conclusions: In presented pilot study we demonstrated clearly relationship between the percentage of immature DCs and the percentage and the phenotype of CTLs in peripheral blood of lung cancer patients.

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Key words: dendritic cells, cytotoxic T lymphocytes, NSCLC, interleukin 12-receptor.

Abbreviation list: DCs – dendritic cells; CTLs – cytotoxic T lymphocytes; NSCLC – non-small cell lung cancer; IL-12R – interleukin 12 receptor; PBDCs – peripheral blood dendritic cells; BDCA – blood dendritic cells antigen.

Introduction

Almost one million of new cases of different types of lung cancer are recorded every year in the world [1]. In Poland, the number of the lung cancer cases systematically increases and the incidence of this disease is about 70 per 100.000 subjects. An increase in the number of cancer patients results not only from the influence of the environmental factors, with a leading strongly negative effect of tobacco smoking, but also of the genetic and immunologic disturbances [1].

More and more investigations refer to the problems in the antitumour immunological response. In response to cancer antigens, dendritic cells (DCs) are the most effective antigen presenting cells [2]. DCs are rare, heterogeneous population of cells that are principally involved in the antigen presentation and stimulation of lymphocytes [2]. Antigenic fragments, after antigens processing in DCs, are exposed at the cell surface through major histocompatibility complex (MHC) responsible for an efficacious antigen presentation [2]. DCs bear costimulatory molecules and possess ability to produce critical cytokines (chemokines, IL-12), thus ensuring the initiation and the fate of acquired immunity [3].

IL-12 stimulates the cell-mediated immune response [4]. This cytokine, in co-operation with IL-2, causes proliferation and activation of Th_1 cells and the cytotoxic activity induction against microbial pathogens and tumour antigens [2,4]. The interaction of NK cells and cytotoxic T lymphocytes (CTLs) with tumour cells involves, at least partly, the binding of the CD95 (Fas/APO-1) antigen present on tumour cells by the CD95 Lig-

Table 1. **Phenotype of lymphocyte in peripheral blood of examined lung cancer patients and in the healthy subjects**

	% granulocyte	% lymphocyte	% CD3+ cells	% CD19+ cells	% CD4+ cells	% CD8+ cells	CD4:CD8 ratio	% NK cells	% CTLs
Lung cancer patients	73.6±9.7%	15.8±7.6%	64.6±10.7%	12.1±8.2%	35.4±11.9%	34.6±10.7%	1.24±0.79	15.4±8.1%	13.1±7.3%
Healthy donors	55.5±7.6	32.2±7.3%	67.6±8.1%	10.9±4.5%	40.2±10.0%	32.1±7.8%	1.31±0.57	14.4±6.3%	10.9±5.5%

and (FasL) present on NK cells and CTLs [5]. The activation of the CD95 antigen results in tumour cell apoptosis [4-7].

Tumour cells are capable of evading the immune system by numerous methods [8]. Tumour-associated macrophages and tumour cells as well as specific type of DCs may secrete immunomodulatory cytokines, such as IL-10, which antagonise the activities of IL-12 [8,9]. IL-10 is a main factor promoting the commitment of naive lymphocytes to Th_2-type profile of cytokine production, which is associated with humoral immune response and immune tolerance [9-11].

The aim of the study was to demonstrate the relationship between percentage of immature dendritic cells and percentage of CTLs subtypes in peripheral blood of non-small cell lung cancer.

Material and methods

The study population comprised of 10 non-small cell lung cancer subjects (the mean age: 61.8±10.55). Five men suffered from squamous lung cancer. The giant cell carcinoma was diagnosed in one man and in one woman. The adenocarcinoma appeared in three men. IIIb stage of lung cancer was diagnosed in five patients, stage IIIa in three patients and stage II in two subjects. The phenotype lymphocytes and CTLs status was also performed in peripheral blood of 22 healthy volunteers.

Peripheral blood samples were collected in heparinised tubes. Mononuclear cells were isolated by density gradient centrifugation on Gradisol L (Aqua Medica, Poland). Interphase cells were removed, washed twice in PBS without Ca^{2+} and Mg^{2+} (Biomed, Poland) containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA (Sigma, Germany) and than resuspended in the mentioned buffer for future immunostaining of DCs.

Three-colour flow cytometry technique was used to establish peripheral blood leukocytes subtypes. The following directly conjugated monoclonal antibodies were used: Simultest (Becton Dickinson), mouse anti-human CD57 FITC, CD8 PerCP, CD19 CyChrome, CD123 PE, CD25 PE, CD69 PE, IL-10 Receptor PE and IL-12 Receptor PE (Becton Dickinson, Pharmingen, USA), CD95Ligand PE (Caltag, USA), BDCA-1 FITC, BDCA-2 FITC (Miltenyi Biotec, Germany). Cells were collected in a FACS Calibur flow cytometer (Becton Dickinson, USA) and their phenotype was analysed using Cell Quest Software. An acquisition gate was established based on FCS and SSC that included all mononuclear cells. Density of antigens on cell surface was determined as mean fluorescence intensity (MFI).

The CTLs were identified as double CD8 and CD57 positive cells. The expression of activation markers and FasL, as well as presence of IL-12 and IL-10 receptor on CTLs was determined. Immunofluorescent staining was performed according to manufacturers' protocol using Lysing Solution (Becton Dickinson, USA). Each measurement contained 30 000 total events.

Myeloid DCs were defined as BDCA-1-positive and CD19-negative cells and lymphoid DCs as BDCA-2 and CD123 double positive cells. Non-specific staining was inhibited by adding 20 μl of FcR Blocking Reagent to 10^7 cells resuspended in the buffer prior the labeling. The cells were incubated for 10 min in the dark at 4°C and washed in the buffer afterwards. Each flow cytometry measurement contained 300 000 total events [12].

Statistical analysis was performed using non-parametric Spearman correlation test, Wilcoxon matched pair test as well as Statistica 5.0 software.

Results

In lung cancer patients group, the percentage of myeloid DCs was slightly higher than the percentage of lymphoid DCs (0.19±0.15% and 0.16%±0.20%, respectively). The myeloid to lymphoid DCs ratio was 3.05±4.40.

The peripheral blood lymphocyte subtypes were represented by the following percentage of cells: T lymphocytes – 64.6±10.7%, B lymphocytes – 12.1±8.2%, T helper cells – 35.4±11.9%, suppressor-cytotoxic T cells – 34.6±10.7%, NK cells – 15.4±8.1%. CTLs CD8 and CD57 double positive cells was 13.10%. 45.7±13.0% of CD8-positive cells had CD57 antigen (*Tab. 1*).

FasLigand was expressed on 13.31±14.42% of CTLs. Activation markers (CD69, CD25) appeared on 18.05±12.32% and 5.02±6.05% of CTLs. Slightly lower percentage of CTLs expressed receptor for IL-12 (65.10±21,87%) than receptor for IL-10 (72.96±17.75%). Expression of FasLigand and receptors for IL-12 as well as IL-10 on CTLs surface was following: 46.84±61.51 MFI, 29.73±10.42 MFI, 31.01±15.89 MFI (*Tab. 2*).

The percentage of granulocyte was significantly ($p<0.005$) higher and the percentage of lymphocyte was significantly ($p<0.005$) lower in PB of lung cancer patients than of healthy donors. The CD4:CD8 ratio was slightly ($p=0.12$) lower in lung cancer group compared to the control group. The percentage of early activated, double positive CD8, CD69 lymphocytes was significantly ($p<0.05$) higher in lung cancer patients than in the control group. There were no significant differences in the percentage of CTLs with IL-2R, CD69 and FasL expression between the lung cancer and the control groups (*Tab. 1, 2*). The expression of FasL on CTLs as well as the percentage of

Table 2. **CTLs status characteristic in the patients with lung cancer and in the healthy donors**

	% CTLs among CD8+ cells	% FasL+ CTLs	Exp. FasL on CTLs (MFI)	% CD69+ CTLs	% CD25+ CTLs	% IL12R+ CTLs	Exp. IL-12R on CTLs (MFI)	% IL10R+ CTLs	Exp. IL-12R on CTLs (MFI)
Lung cancer patients	45.7±13.0%	13.31±14.42%	46.84±61.51	18.05±12.32%	5.02±6.05%	65.10±21.87%	29.73±10.42	72.4±18.2%	31.01±15.89
Healthy donors	40.6±14.3%	15.8±12.0%	29.9±26.8	20.2±15.9%	3.9±3.9%	70.8± 20.7%	28.51±8.22	79.7±17.5%	36.44±17.37

Figure 1. **Correlation between percentage of lymphoid DCs and percentage of CTLs in peripheral blood of lung cancer patients**

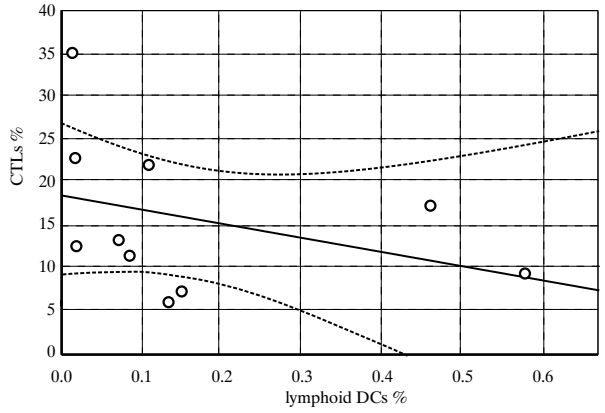
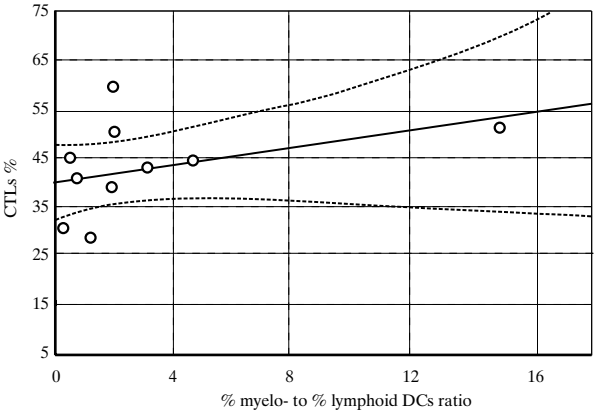


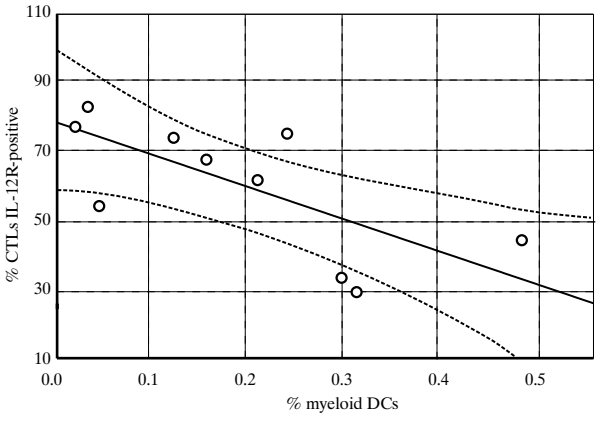
Figure 2. **Correlation between myeloid- to lymphoid DCs ratio and percentage of CTLs in peripheral blood of lung cancer patients**



CTLs with IL12R and IL10R were slightly higher in lung cancer patients than in healthy subjects.

Significant negative correlation ($R=-0.693$, $p<0.05$) between the percentage of lymphoid DCs and the percentage of CTLs was shown (*Fig. 1*). The myeloid to lymphoid DCs ratio significantly positively ($R=+0.638$, $p<0.05$) correlated with the percentage of CTLs (*Fig. 2*). The significant negative correlation between the percentage of myeloid DCs and the percentage of CTLs with IL-12R (*Fig. 3*) as well as expression of this receptor were also ascertained ($R=-0.68$, $p<0.05$ and $R=-0.757$, $p<0.01$, respectively).

Figure 3. **Correlation between percentage of myeloid DCs and percentage of CTLs with expression of IL-12R in peripheral blood of lung cancer patients**



Discussion

Dendritic cells are highly effective antigen-presenting cells [14]. They are raised from hematopoietic stem cells in the bone marrow and are widely distributed as immature DCs into both lymphoid and non-lymphoid tissues [15,16]. The potential mechanisms controlling the production of DCs or their progenitors within bone marrow are not known [2,16,17]. Nor it is known whether these putative controls are autonomous, exclusive of bone marrow, or dependent on exogenous-paracrine influences, or affected by external environmental stimuli like microbial products [2].

The DCs population represents only a minute subpopulation (less than 1%) of the peripheral blood mononuclear cells [12,18]. The recent studies have demonstrated that in human peripheral blood exist at least two subpopulations of circulating immature dendritic cells: myeloid and lymphoid subsets [13,18,19]. These two subpopulations of PBDCs (peripheral blood dendritic cells) are characterized by expression of specific markers for circulating DCs – blood dendritic cells antigens: BDCA-1 and BDCA-2 [12,13]. BDCA-1, which in cluster differentiation correspond to CD1c antigen, is expressed on human myeloid subpopulation of PBDCs [12]. BDCA-1-possitive cells express myeloid markers and secrete IL-12 after stimulation of foreign antigens [12]. BDCA-2 is specifically expressed on human plasmacytoid (lymphoid) dendritic cells and it is postulated that BDCA-2-positive cells produce a large amount of IL-4 after antigen stimulation [13]. These two subsets of peripheral blood dendritic cells were claimed to stimulate Th1 and Th2 types of immune responses,

respectively, and hence, were termed by some investigators as DC1 and DC2 [12,16]. However, other reports suggest that differentiation of naïve T cells is also determined by cytokine environmental (IL-12 versus IL-4) and the activation state of DCs [16,19].

Our recent investigation confirmed that the percentage of both myeloid and lymphoid DCs was lower in patients with NSCLC than in the healthy subjects. Furthermore, percentage of immature DCs in peripheral blood of cancer patients may correspond to the type of neoplasm [20]. In another study, we showed expression of IL-12R and IL-10R on peripheral blood CTLs in lung cancer patients. Percentage of IL-12R-positive cells was lower in lung cancer patients than in the healthy persons. The phenotype of CTLs was depended on immunological system status (presence of inflammation process) and clinical manifestation of the disease [10,21,22].

Our observation presented in this paper is a pilot study on the quantitative analysis of dendritic cells and allows us to conclude that the connection between percentage of immature DCs and CTLs appeared in peripheral blood of lung cancer patients. We affirmed negative correlation between percentage of myeloid DCs and percentage of CTLs with expression of IL-12 receptor. We hypothesised, that this relationship is in connection with compartmentalisation of immunological response in lung cancer. In NSCLC patients, myeloid DCs may migrate from peripheral blood to tumour tissue and metastatic lymph nodes. Mature DCs in lymph nodes could produce IL-12 and stimulate CTLs. However, it is postulated that the mature DCs may be malfunctioning in the cancer-bearing patients [8,22].

The correlation between percentage of DCs and percentage of CTLs may be also independent phenomenon. In this coincidence, numerous of factors connected with development of lung cancer may posses the independent ability to influence on percentage of bone marrow-derived DCs and CTLs [8]. Third hypothesis is based on the data, that not only mature DCs seeded in tissues and lymph organs, but also immature DCs in peripheral blood could process the tumour antigens and produce IL-12 [9]. Associated with mentioned facts, we concluded that direct relationships between percentage of immature DCs and phenotype and percentage of CTLs in peripheral blood of NSCLC patients may exist. Therefore, establishing these correlation is still an open question and further studies are clearly needed to examine these relationships.

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Correlation of B type natriuretic peptides with clinical and echocardiographic parameters in heterogeneous population of patients with symptoms suggestive of heart failure

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Abstract

Purpose: NT-proBNP and BNP concentrations in CHF correlate with NYHA class and LVEF. Little research has been conducted to compare the clinical performance of these two natriuretic peptides in heterogeneous CHF population. Purpose: to evaluate and compare the clinical performance of NT-proBNP and BNP in heterogeneous group of CHF patients on the basis of these peptides' correlation with NYHA class, LVEF and WMI measured by echocardiography.

Material and methods: Consecutive patients admitted for suspected of CHF. Blood samples were drawn for NT-proBNP, BNP, creatinine and echocardiography was performed.

Results: 71 patients were included. CHF was diagnosed in 53. Sensitivity of NT-proBNP and BNP in diagnosing CHF was 83% and 94% respectively (P=0.079). Levels of both peptides correlated equally well with NYHA class (R=0.537, p<0.001; R=0.473, P<0.001), LVEF (R=-0.623, p<0.001; R=-0.601, P<0.001) and WMI (R=0.590, P<0.001; R=0.527, P=0.001). Creatinine correlated with both peptides, age correlated with NT-proBNP. No difference between sexes was found in both peptides' concentrations. In multivariate analysis independent determinants of BNP were LVEF, presence of valvular disease and NYHA class. In case of NT-proBNP age and creatinine also displayed independent influence.

Conclusions: NT-proBNP and BNP show good sensitivity in detecting CHF. Levels of both peptides correlate equally well with clinical and echocardiographic parameters of CHF, which makes them equally adequate in biochemical staging of CHF's severity regardless of its underlying cause. Levels of natriutretic peptides reflect contractile dysfunction, valvular disease and clinical condition. Age and creatinine concentration but not patients' sex should additionally be considered when measuring NT-proBNP.

Key words: NT-proBNP, BNP, chronic heart failure, NYHA class, LVEF, valvular heart disease.

Introduction

Tests measuring B type natriuretic peptides (BNP, brain natriuretic peptide and its biologically inactive counterpart – NT-proBNP, N-terminal probrain natriuretic peptide) have established themselves as diagnostic as well as prognostic tools in chronic heart failure and acute coronary syndromes. [1] In heart failure BNP and NT-proBNP are produced in increased quantities from the common precursor, pre-pro-BNP, and released from the ventricles in response to increased myocardial stretch and elevated ventricular pressure. The levels of B type natriuretic peptides depend on the amount of ventricular wall stretch and the severity of cardiac damage. Therefore their concentrations correlate with the clinical condition (NYHA class) and systolic function of the left ventricle (left ventricle ejection fraction, left ventricle end diastolic diameter, left ventricle end diastolic volume) measured by echocardiography, radionuclide scintigraphy or magnetic resonance imaging despite certain discrepancies in the strength of these correlations [2-4].

In establishing diagnosis and prognosis in patients suspected of heart failure tests measuring NT-proBNP and BNP show similar performance [5,6] in spite of some differences between the two analytes in half-life in circulation, *ex vivo* stability, renal clearance, age and sex with NT-proBNP being more stable, having longer half-life and depending more on renal function and age, which may be caused by its renal non-receptor mediated elimination [7,8]. It is not quite clear; however, if levels of both peptides perform equally well in clinical practice in a population with various underlying causes of heart dysfunction since there

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Table 1. **Clinical characteristics of the patients with chronic heart failure (CHF) and without chronic heart failure (non CHF)**

Patient group	n	MI	VD	DCM	CAD non-MI	AH	LVEF* (%)	Crea* (mg/dl)	PE
All	71	25	12	11	9	11	48.1 (25-73)	1.2 (0.6-4.1)	1
CHF (S/D)	53	21 (21/0)	11	11 (11/0)	5 (4/1)	4 (1/3)	43.9 (25-73)	1.3 (0.6-4.1)	1**
Non-CHF	18	4	1	0	4	7	60.2 (46-70)	1.05 (0.7-1.5)	0

AH – arterial hypertension; CAD non-MI – coronary artery disease without myocardial infarction; CHF – chronic heart failure; DCM – dilated cardiomyopathy; MI – myocardial infarction; Non-CHF – no chronic heart failure (heart failure excluded); PE – pulmonary embolism; VD – valvular disease; S/D – systolic (mainly)/diastolic dysfunction; LVEF (%) – left ventricle ejection fraction; Crea – creatinine concentration; * mean and range; ** right-sided heart failure

are only few such head-to-head comparisons of these markers. [9,10].

The aim of the study

The aim of the study was to evaluate and compare the clinical performance of NT-proBNP and BNP in patients with symptoms suggestive of heart failure and various cardiovascular conditions predisposing to it on the basis of correlations of these peptides' levels with clinical condition (NYHA class) and echocardiographic parameters (LVEF, WMI).

Material and methods

We included consecutive patients admitted to the department for symptoms suggestive of heart failure. On the second day of hospitalization all patients underwent physical examination preceded by detailed history taking independently of the first examination performed on admission. On the same day echocardiography was performed and blood sample was obtained for NT-proBNP, BNP and creatinine measurement. Diagnosis of heart failure was made on the basis of clinical evaluation (Framingham criteria) [11] and echocardiography: symptoms of dyspnea in accordance with Framingham criteria and sings of pulmonary and/or peripheral congestion accompanied by relevant echocardiographic findings. All patients received optimal treatment. The patients' clinical condition was evaluated and they were divided into NYHA classes I-IV. The echocardiographic examination was performed with a Vingmed ultrasonograph by a single echocardiographer blinded to peptide values and exact clinical characteristics of patients. LVEF (left ventricle ejection fraction) was evaluated and calculated by the modified Simson's formula. WMI (wall motion index) was calculated according to the 16-segment division of the left ventricle. The anatomy of the valves was evaluated in 2-D mode and flow, gradients and regurgitation with pulsed-wave, continuous wave and color Doppler. The presence of a significant valvular disease was established according to commonly accepted criteria [12,13]. NT-proBNP concentration was measured in serum with Roche Elecsys 2010 analyzer. BNP concentration was measured in plasma with Abbott AxSYM analyzer. Creatinine concentration was measured with Roche Integra 700 analyzer.

Bioethics committee of the Military Medical Institute approved the study.

Table 2. **Concentrations of BNP and NT-proBNP in patients with and without CHF**

	Non-CHF (n=18) (median and range)	**CHF** (n=53) (median and range)	**P**
BNP (pg/mL)	47 (1.5 – 485)	297 (0 – 3353)	<0.001
NT-proBNP (pg/mL)	245 (35 – 1387)	2353 (13 – 42034)	<0.001

Since the distribution of natriuretic peptide concentrations was not normal, results were shown as medians and ranges. The differences in values between groups were estimated with a U Mann-Whitney test. The difference in diagnostic sensitivity of the two peptides was estimated with an index of structure difference. Correlations of natriuretic peptide levels with other parameters were estimated with a non-parametric Spearman method or with Pearson method after logarithmic transformation leading to normalization of value distribution. The latter was especially done to normalize distribution before calculating and comparing differences between correlation coefficients. In order to elicit the independent determinants of B type natriuretic peptides' levels multivariate analysis was performed that included all significant variables and confounding factors.

Results

The study included 71 patients (39 men and 32 women aged between 34 and 94, mean age 67 years). Chronic heart failure diagnosis was confirmed in 53 patients (75%). Three patients were in NYHA class I, 26 in NYHA class II, 18 in class III, and 6 in class IV.

Detailed characteristics of patients including the underlying cause of chronic heart failure or other cardiovascular diseases is presented in *Tab. 1*.

NT-proBNP and BNP concentrations were significantly higher in the CHF group compared with the non-CHF group. Median concentration of BNP was 6.3 times higher and NT-proBNP was 9.6 times higher in the CHF group compared with the non-CHF group (*Tab. 2*).

The roughly estimated diagnostic sensitivity in detecting heart failure was 83% for BNP (cut-off value of 100 pg/ml) and 94% for NT-proBNP (cut-off value of 125 pg/ml) (diagnostic cut-offs recommended by manufacturers). The difference

Table 3. **Correlations of natriuretic peptide concentrations with NYHA class, echocardiographic parameters, creatinine concentration and age in patients with CHF and in all patients (italics)**

	Number of pts	NYHA	LVEF[#]	WMI[&]	Creatinine	Age
BNP	53	0.473***	-0.452***	0.423*	0.332*	0.226
(pg/mL)	71		*-0 .601****	*0.527****	*0.387****	*0.292**
NT-proBNP	53	0.537***	-0.421**	0,443**	0.443***	0.345*
(pg/mL)	71		*-0.623****	*0,590****	*0.473****	*0.384***

[#] LVEF – Left Ventricle Ejection Fraction; [&] WMI – Wall Motion Index; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

between the sensitivities was not statistically significant but it was not far from it (P=0.079).

Concentrations of natriuretic peptides correlated significantly with NYHA class. NT-proBNP tended to correlate with NYHA class better than BNP but correlation coefficients were not significantly different (*Tab. 3*).

NT-proBNP and BNP correlated similarly with echocardiographic parameters (LVEF, WMI). NT-proBNP tended to correlate stronger with creatinine concentration than BNP but this difference was not significant. Correlations of B type natriuretic peptides' levels with echocardiographic parameters were stronger for a wider range of values (i.e. when all, CHF and non-CHF patients were included) than for a narrower range typical of patients with CHF (*Tab. 3*).

Concentrations of NT-proBNP and BNP correlated well with each other for all patients (R=0.899; P=0.001) and for CHF patients (R=0.879; P<0.001).

In patients with CHF BNP levels were not dependent on age and NT-proBNP levels increased with age mildly. We did not observe any significant differences in NT-proBNP and BNP concentrations between men and women in the whole group or in the group with CHF [log NT-proBNP (all men/CHF men)= 3.163 (0.704)/3.322 (0.627), log NT-proBNP (all women/CHF women)=3.010 (0.840)/3.376 (0.753), P=0.41/P=0.78; log BNP (all men/CHF men)=2.320 (0.580)/2.442 (0.539), log BNP (all women/CHF women)=2.211(0.939)/2.687 (0.611) P=0.14/P=0.56]. In our patients creatinine concentrations did not depend on sex and age.

In multivariate analysis the levels of NT-proBNP and BNP were independently determined by LVEF, the presence of significant valvular disease and NYHA class. Furthermore, age and renal function were also independent determinants of NT-proBNP (*Tab. 4 a,b*).

Table 4. **Multiple regression analysis of factors influencing NT-proBNP (4a) and BNP (4b) concentrations**

4a)

	BETA	Standard error	P value
LVEF	-0.391	0.090	<0.001
VD	-0.246	0.010	0.017
NYHA	0.245	0.104	0.022
Age	0.260	0.092	0.007
log10 Crea	0.237	0.096	0.018

4b)

	BETA	Standard error	P value
LVEF	-0.509	0.103	<0.001
VD	-0.411	0.113	<0.001
NYHA	0.237	0.116	0.049
log10 Crea	0.044	0.109	0.686

LVEF – left ventricle ejection fraction; VD – valvular disease, NYHA – New York Heart Association class; Crea – creatinine

Discussion

The concentration of both peptides (NT-proBNP and BNP) were significantly higher in patients who were diagnosed with heart failure. The B type natriuretic peptides detected heart failure with a good sensitivity exceeding 80% at cut-offs recommended by the manufacturers. Such high sensitivity could partially be explained by the fact that almost half of the examined patients were severely ill (NYHA class III and IV). The average increase in NT-proBNP in CHF patients was 50% greater than that of BNP. This could be a basis for a hypothesis, partially supported by other trials, that NT-proBNP could be slightly more sensitive in detecting heart failure and might be a slightly better discriminator among NYHA classes than BNP. Our study showed that NT-proBNP tended to correlate with NYHA class better that BNP and was slightly (but not significantly) more sensitive. This is consistent with findings by other authors [9,10].

Concentrations of NT-proBNP and BNP correlated significantly with NYHA class, LVEF and WMI measured by echocardiography. Correlations of these peptides with echocardiographic parameters were practically identical, which remains consistent with findings by Masson et al. who demonstrated clinical equality of BNP and NT-proBNP [14].

Concentrations of both natriuretic peptides did not depend on sex in our patient group. Apparently mechanisms involved in synthesis and secretion of natriuretic peptides in CHF exert such a strong influence on their levels that difference between the sexes in peptide levels observed in healthy population becomes insignificant [15]. This hints at the possibility of adopting one diagnostic cut-off independent of sex. The levels of both peptides depended on creatinine concentration and this correlation was slightly stronger for NT-proBNP. This observation reflects the results of other studies [16].

This more pronounced dependence of NT-proBNP levels on renal function may result from the fact that NT-proBNP is cleared mainly by kidneys in an unchanged form, whereas there is an additional receptor-dependent path of BNP elimination [17]. It must be stressed; however, that in our patient group only 2 persons had creatinine levels exceeding 2 mg/dl.

NT-proBNP showed a weak correlation with age which was independent of creatinine.

The multivariate analysis showed that left ventricle ejection fraction, the presence of valvular disease and NYHA class were the main independent determinants of NT-proBNP and BNP levels. Moreover, the levels of NT-proBNP were also independently influenced by age and creatinine concentration. This is an important finding demonstrating that contractile dysfunction, valve dysfunction and present clinical status (level of exacerbation) all significantly contribute to the elevated NT-proBNP/BNP levels we measure. Few studies have so far tried to ascribe natriuretic peptides' levels to particular clinical and echocardiographic parameters in heterogeneous group of patients and compare the two commercially available markers in this respect since most trials concentrated on comparative analytical precision and sensitivity analysis [9,10,18,19]. NT-proBNP levels also reflect renal function and aging and these two factors need to be considered when this marker is measured. With this exception the clinical usefulness of both markers is comparable and they can both serve as credible gauges of cardiac performance in HF patients.

The group examined in our study was heterogeneous. The common denominator for all participants was the presence of cardiovascular disease having already caused or predisposing to heart failure in the future. According to the new heart failure classification these patients belong to group A, B, C or D [20]. Clinically symptomatic heart failure in the traditional sense had 53 out of 71 participants (groups C and D of the new classification). Such choice of patients, far from optimal from the methodological standpoint, and their limited number is undoubtedly a limitation to the study. On the other hand it reflects the working conditions of a clinician and falls in line with an accepted method of including consecutive patients in a study.

Conclusions

1. Both B type natriuretic peptides (BNP and NT-proBNP) demonstrate good sensitivity in detecting heart failure which exceeds 80%.

2. Concentrations of NT-proBNP and BNP correlated well both with clinical condition of the heart failure patients (NYHA class) and echocardiographic parameters of left ventricle dysfunction (LVEF, WMI). This makes the two peptides equally adequate for the evaluation of the severity of heart failure regardless of its underlying cause.

3. Contractile left ventricle dysfunction, valve dysfunction and present clinical status all significantly contribute to both NT-proBNP and BNP levels in heterogeneous population of heart failure patients.

4. Levels of B-type natriuretic peptides do not depend significantly on sex in chronic heart failure patients so this factor need not be considered in diagnosing or monitoring heart failure patients with NT-proBNP/BNP. On the other hand age and renal function need to be considered especially when interpreting NT-proBNP results.

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Determinants of nutritional status of older people in long-term care settings on the example of the nursing home in Białystok

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Abstract

Purpose: The aim of the study was the assessment of the nutritional status of older people living in the chosen long-term care setting in Poland as well as the determinants having an effect on the nutritional status of the examined subjects.

Materials and methods: The subjects included older residents (aged 65 years and older) of the nursing home for the somatically ill adults in Bialystok. The MNA-Mini Nutritional Assessment test was used as an assessment tool to detect nutritional risk. The assessment included elements of clinical and functional evaluation (Katz Index, Instrumental Activities of Daily Living (ADL) scale, Geriatric Depression Scale, Abbreviated Mental Test Score, Norton scale and mobility scale).

Results: One hundred out of the 109 persons fulfilling the age criterion were examined. We found that 12% of them were malnourished, 61% were at risk of malnutrition and 27% were well nourished according to the MNA test. Malnutrition affected more often persons having difficulties with chewing, ADL dependent, with limited mobility, suspected of dementia, having suffered from cerebral stroke and who lived with other people coming to the nursing home. The risk of malnutrition was observed significantly more often in individuals suspected of depression and living in urban area before nursing home placement. The significant determinants of lower scores of MNA in the regression analysis were: suspected depression, IADL dependency, limited mobility, female gender and higher number of drugs.

Conclusions: The study has confirmed that malnutrition remains a common problem among older people living in nursing homes. Malnutrition is an increasing hazard especially for women, for people taking higher number of drugs and for those with different mental and physical disabilities.

Key words: malnutrition; older people in nursing home; Mini Nutritional Assessment.

Introduction

Aging does place individuals at a greater nutritional risk [1,2] but the prevalence of malnutrition reaches significant levels first of all in elderly patients who are in hospitals or who live in nursing homes [3]. The prevalence of protein-energy malnutrition in nursing home residents ranges from 23 to 85% [4]. Poor nutritional status is related to increased morbidity, and malnutrition is associated with an increased incidence of morbidity and mortality [5].

The diagnosis of protein-energy malnutrition in elderly populations is difficult. There is no generally accepted "gold standard" for diagnosis and precise nutritional assessment is difficult in everyday clinical practice [6,7]. A brief nutritional assessment test such as the Mini Nutritional Assessment (MNA) could be an effective way of screening patients who are undernourished. The aim of the MNA is to evaluate an individual's risk of malnutrition so as to permit early nutritional intervention when necessary [8].

The aim of the study was the assessment of the nutritional status of older people living in the chosen long-term care setting in Poland as well as the determinants having an effect on the nutritional status of the examined subjects. The paper attempts also to find determinants of nutritional status of older people living in nursing home assessed with Mini Nutritional Assessment scale.

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Material and methods

The subjects included older residents (aged 65 years and older) of the nursing home for the somatically ill adults in Bialystok. One hundred out of the 109 persons fulfilling the age criterion were examined. The response rate was 91.7%. 9 individuals did not want to participate or were not able to participate because of their serious health status.

A questionnaire taking into account the following: 1) the social-demographic evaluation, 2) the experiences connected with living in nursing home, 3) health status with elements of clinical judgment, the occurrence of chronic illnesses, and medication, 4) the functional evaluation of the respondent with functional ability scales, and 5) nutritional status assessment with Mini Nutritional Assessment Scale (MNA) – was used.

The following instruments were used to rate the respondent's functional ability:

- The Katz Index [9] – used to evaluate functional ability in the range of basic ADL (P-ADL – personal activities of daily living) – being able to take care of oneself (eating, incontinence, using the toilet, bathtub/shower, dressing, moving). It placed individuals into one of three groups: functionally able – 5-6 points; moderately ADL dependent – 3-4 points, and severely ADL dependent- 1-2 points.
- Instrumental Activities of Daily Living scale (I-ADL) [10]. This took into account instrumental activities of daily living, such as cleaning, meals preparation, shopping, using the telephone, taking medication, moving outside the home etc. If the respondent was able to perform the activity without any help he or she was given 3 points, with some help – 2 points, and not able at all – 1 points (the answers can give maximum 27 points).
- The scale of mobility according to Piotrowski [11] – placed the respondents into one of four groups. Group I – persons able to walk freely at home and outside the home; group II – persons walking freely around the home but having difficulties moving outside the home; group III – persons able to walk around the home but who can not move outside the home; group IV – persons who are bedridden, in a wheelchair, or confined to an armchair.
- A questionnaire rating cognitive functions – Abbreviated Mental Test Score (AMTS) [12] – the test results were on the following scale: from 0 to 3 points showed a serious cognitive impairment, from 4 to 6 points showed a moderate cognitive impairment, above 6 points (maximum 10 points) normal state of cognitive functions in the respondent.
- The Geriatric Depression Scale (GDS) [13] – graded the emotional state of the respondent in two stages: from 0 to 5 points as a normal emotional state and a suspected state of depression with a rising tendency from 6 to 15 points.
- Norton scale assessing risk of developing pressure sores [14] – 14 points or above pointed to the normal status and less then 14 points – to the risk of pressure sores.

The Mini Nutritional Assessment (MNA) test [15] was used as an assessment tool to detect nutritional risk. The instrument consists of 18 point weighted questions in four categories (*Tab. 1*). The first category consists of four anthropometric measurements such as body mass index (BMI), mid upper arm and calf circumferences and weight loss during the past 3 months. Six global questions regarding accommodation type, pharmaceutical consumption, acute diseases (including psychological stress), mobility, neuropsychological problem, and pressure sores/skin ulcers constitute the second category. The third part consists of six questions assessing dietary intake and the final part of two self-assessments of whether food intake is sufficient and of the own health status. The answers can give maximum 30 points. Less than 17 points is regarded as indicated malnutrition, 17-23.5 points indicate a risk of malnutrition, and ≥24 points indicate that the person is well nourished.

Table 1. **Elements of MINI NUTRITIONAL ASSESSMENT (MNA) [15]**

I.	**Anthropometric assessment**	• BMI – body mass index • mid upper arm and calf circumferences • weight loss during the past 3 months
II.	**Global evaluation**	• accommodation type – living independently or in nursing home, • taking more then 3 prescription drugs, • psychological stress or acute disease in the past 3 months, • mobility, • neuropsychological problems, • pressure sores or skin ulcers
III.	**Dietetic assessment**	• quantity and quality of eating meals, • loss of appetite, • digestive problems, chewing or swallowing difficulties causing decline in patient food intake, • beverages consumed per day, • mode of feeding
IV.	**Subjective assessment**	• Does patient consider to have any nutritional problems? • How would the patient consider his health status in comparison with other people of the same age?

Two experienced and trained in geriatric assessment nurses, not employed in the studied nursing home, were interviewers.

The STATISTICA 7.0 [16] program was used to analyze the collected data. In case the Chi square Pearson test was used a p-value of 0.05 or lower was considered to be statistically significant.

Multivariate regression analysis (standard, stepwise backward and forward) was used to find independent variables influencing nutritional status of the group studied. The total score of MNA was used as the dependent variable for multiple regression analysis. The multivariate regression model was constructed with independent variables presented in *Tab. 2*, describing both the general situation of the older person as well as his or her functional abilities. The variables previously associated with the risk of malnutrition from the literature were chosen. Some of the variables were constituted of the total scores on different scales as described above. First of all the variables correlating significantly with the total score of MNA were chosen. The analysis was done in stages. Standard regression was used to assess the relationships among variables in the model. To eliminate independent variables those do not provide additional prediction to the variables already in the equation stepwise backward regression was used. The stepwise forward regression was used to check the proportion of variance attributable to

Table 2. **Variables included in the multivariate regression models analyzing chosen determinants of the Mini Nutritional Assessment score**

Variable	Label/Value (in case of scales scores – min-max; mean in the group studied)
I. Socio-demographic characteristics	
Age	number of years (66-102; 79.1)
Gender	female=0 male=1
Living alone before nursing home placement	yes=0 no=1
II. Functional abilities	
Katz Index (ADL) score	total score on the scale (0-6; 3.97)
IADL score	total score on the scale (9-27; 14.6)
GDS score	total score on the scale (0-14; 7.66)
AMTS score	total score on the scale (0-10; 7.0)
Norton scale score	total score on the scale (10-20; 15.8)
Piotrowski mobility scale score	I/ II group of mobility =0 III/IV group of mobility =1
III. Other characteristics	
Number of drugs taken every day	total number of drugs (0-13; 4.4)
Stroke in the past	without =0 yes =1
Difficulties in chewing	no =0 yes =1
Place of having meals	a resident's room =0 a dinner room =1

ADL – Activities of Daily Living; IADL – Instrumental Activities of Daily Living; GDS – Geriatric Depression Scale; AMTS – Abbreviated Mental Test Score

some independent variables after variance due to variables in equation is accounted for.

The study was approved by the Ethics Committee of the Medical University of Białystok.

Results

Age and gender of the examined groups is presented in *Tab. 3*. Most of the respondents were women, belonging to the older subgroup. The average age was 80.4 years (±7.79) in women and 75.9 (±6.86) in men. Differences in age structure between male and female group were statistically significant (p=0.007 in Chi square Pearson test).

We found that 12% of the group members were malnourished (<17 points), 61% were at risk of malnutrition (17-23.5 points) and 27% were well nourished (≥24 points) according to the MNA test (*Fig. 1*). The distribution of malnutrition and risk of malnutrition in different subgroups of the studied individuals are presented in *Tab. 4*, and *Tab. 5*.

Well nourished individuals were observed significantly more frequently in men (p<0.05), people with good psycho-physical abilities – good mobility (p<0.001), without signs of depression (p<0.001) or cognitive impairment (p<0.001), ADL independency (p<0.01), no risk of pressure sores (p<0.05), with good teeth status (p<0.01), having meals in a dinning room, and not in the resident's room (p<0.001). The risk of malnutrition was observed significantly more frequently in the group of older people living in urban area before nursing home placement (p<0.05), with suspected depression (p<0.01). Malnutrition affected significantly more frequently subjects living with other people before nursing home placement (p<0.05), having meals

Table 3. **Age and gender structure of examined group**

Gender	Males		Females		Total
	n	%	n	%	n=%
Age*					
65-74 years old	14	48.3	15	21.1	29
+75 years old	15	51.7	56	78.9	71
Total	29	100.0	71	100.0	100
Average age [n years; ±SD]	75.9 (±6.86)		80.4 (±7.79)		79.1 (±7.78)

SD – standard deviation

Figure 1. **The prevalence of malnutrition, and risk of malnutrition in older residents of studied nursing home (N=100)**

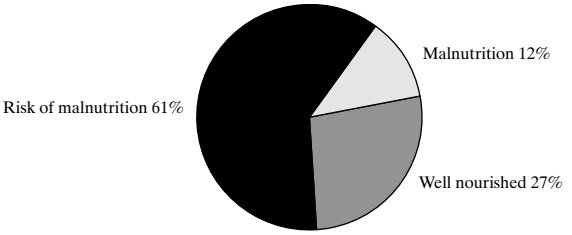

Table 4. **Nutritional status according to Mini Nutritional Assessment (MNA) and age, gender and living arrangements (N=100)**

Nutritional status according to MNA		Well nourished [%]	Risk of malnutrition [%]	Malnutrition [%]
Gender				
Female (n=71)		21.1	63.4	15.5
Male (n=29)		41.4	55.2	3.4
	p*	<0.05	NS	NS
Age Group				
65-74 years old (n=29)		31.0	62.1	6.9
+75 years old (n=71)		25.3	60.6	14.1
	p*	NS	NS	NS
Place of living before nursing home placement				
Urban (n=83)		11.8	82.3	5.9
Rural (n=17)		30.1	56.6	13.3
	p*	NS	<0.05	NS
Alone living before nursing home placement				
Yes (n=41)		29.3	68.3	2.4
No (n=59)		25.4	55.9	18.7
	p*	NS	NS	<0.05
The length of stay in nursing home				
<12 months (n=24)		25.0	58.3	16.7
+1 year (n=76)		27.6	61.9	10.5
	p*	NS	NS	NS
Place of having meals in nursing home				
Resident's room (n=64)		15.6	65.6	18.8
Dinning room (n=36)		47.2	52.8	0.0
	p*	<0.001	NS	<0.01

* – two tailed U test for two frequencies

Table 5. **Nutritional status according to Mini Nutritional Assessment (MNA) and functional/health status (N=100)**

Nutritional status according to MNA	Well nourished [%]	Risk of malnutrition [%]	Malnutrition [%]
Mobility (Piotrowski scale)			
Group I/II (n=53)	43.4	52.8	3.8
Group III/IV (n=47)	8.5	70.2	21.3
p*	<0.001	NS	<0.01
Cognitive functions			
Normal state (n=55)	41.8	54.6	3.6
Suspected dementia (n=44)	8.9	68.9	22.2
p*	<0.001	NS	<0.01
Emotional status			
Normal state (n=28)	57.1	39.3	3.6
Suspected depression (n=72)	15.3	69.4	15.3
p*	<0.001	<0.01	NS
ADL functional ability			
Functionally able (n=58)	37.9	60.4	1.7
ADL dependent (n=42)	11.9	61.9	26.2
p*	<0.01	NS	<0.001
Risk of pressure sores (Norton scale)			
Risk (n=27)	11.1	51.9	37.0
No risk (n=73)	32.9	64.4	2.7
p*	<0.05	NS	<0.0001
Self-rating of the health status			
Good (n=25)	40.0	52.0	8.0
Average/poor (n=75)	22.7	64.0	13.3
p*	NS	NS	NS
Chewing difficulties			
No/little (n=87)	29.9	63.2	6.9
Yes, big difficulties (n=13)	7.7	46.2	46.1
p*	NS	NS	<0.001
Status of teeth			
Good status (n=24)	50.0	45.8	4.2
Poor dentition (n=76)	19.7	65.8	14.5
p*	<0.01	NS	NS
Stroke in the past			
No (n=88)	29.5	61.4	9.1
Yes (n=12)	8.3	58.3	33.4
p*	NS	NS	<0.05
Number of drugs taken			
0-3 drugs (n=40)	32.5	65.0	2.5
+4 drugs (n=56)	25.0	57.1	17.9
p*	NS	NS	<0.05

ADL – Activities of Daily Living; * – two tailed U test for two frequencies

in resident's room at nursing home ($p<0.01$), with different psycho-physical dysfunctions – pore mobility ($p<0.01$), suspected dementia ($p<0.01$), ADL dependency ($p<0.001$), risk of pressure sores ($p<0.0001$), with chewing difficulties ($p<0.001$), having suffered from cerebral stroke in the past ($p<0.05$) and taking 4 or more drugs every day since 3 months ($p<0.05$). There were no significant differences observed between individuals in different age groups, with different lengths of stay at nursing home, rating differently their health status.

The average value of MNA in the whole group was 21.2 [standard deviation ±3.7; minimum – 12.5; maximum – 28.5; kurtosis – (-).066; skeweness – 0.01]. The distribution of MNA scores in our group was normal according to the Lilliefors and Kolmogorov-Smirnov tests of normality.

Table 6. **Standard multivariate regression analysis of independent factors associated with the total score on Mini Nutritional Assessment Scale** (N=96; adjusted $R^2=0.6121$; SE=2.3; F=12.530; $p<0.00001$)

Independent variable	B	S.E.	β	p
Constant*	20.21	3.9		<0.00001
I. Socio-demographic characteristics				
Age	0.013	0.04	0.03	NS
Gender*	1.53	0.59	0.19	<0.05
Living alone before nursing home placement	-0.71	0.52	-0.09	NS
II. Functional abilities				
Katz Index score	-0.034	0.25	-0.02	NS
IADL score *	0.15	0.08	0.21	NS
GDS score *	-0.38	0.08	-0.36	<0.0001
AMTS score	0.08	0.11	0.07	NS
Mobility scale*	-1.59	0.66	-0.21	<0.05
Norton scale score	0.15	0.16	0.12	NS
III. Other characteristics				
Number of drugs taken every day	-0.20	0.08	-0.17	<0.05
Stroke in the past	0.34	0.87	0.03	NS
Difficulties in chewing	-1.02	0.78	-0.09	NS
Having meals in resident's room	-0.47	0.67	-0.06	NS

IADL – Instrumental Activities of Daily Living; GDS – Geriatric Depression Scale; AMTS – Abbreviated Mental Test Score; adjusted R^2 – the adjusted squared multiple correlation; B – denotes the unstandardized coefficient; SE – the standard error of B; β – the standardized coefficient beta; p – the probability value; NS – not significant; * – variables significant in stepwise backward regression of the model

As the distribution of MNA scores in the studied group was normal the multivariate regression analysis was used to find determinants of MNA in nursing home inhabitants. The total score of MNA was used as the dependent variable and the variables described in *Tab. 2* were included in the model as the independent ones. The results of the standard multivariate regression analysis of the model are presented in *Tab. 6*. Together the independent variables included in the model accounted for 61.2% of the total variance of MNA (N=96, adjusted $R^2=0.6121$, SE 2.33, F=12.53, $p<0.00001$). Variables significant in stepwise backward regression of the model are marked with asterisk – together they accounted for 59.8% of the total variance of dependent variable (adjusted $R^2=0.5979$). Lower score on MNA was independently associated with lower IADL scores, higher GDS scores, female gender and difficulties in mobility (III/IV group according to Piotrowski scale).

The stepwise forward regression was used to check the proportion of variance attributable to independent variables after variance due to variables in equation is accounted for. *Tab. 7* presents the consecutive steps of this analysis. Five of the variables have added the significant proportion of variance explanation (together in the analysis – 62.9%). Above the variables essential in stepwise backward regression analysis another important variable has emerged here. It was the number of drugs taken every day – the higher the number the lower MNA score.

Table 7. **Stepwise forward multivariate regression analysis of independent factors associated with the total score on Mini Nutritional Assessment Scale** (N=96; adjusted R^2=0.6287; SE=2.28; F=21.107; p<0.00001)

Independent variable	Step	B	S.E.	β	p	Adjusted R^2
Constant		20.83	2.26		<0.000001	
Norton scale score	1	0.15	0.13	0.12	NS	0.3959
GDS score *	2	-0.40	0.08	-0.38	<0.00001	0.4743
IADL score *	3	0.19	0.07	0.26	<0.01	0.5249
Gender *	4	1.53	0.55	0.19	<0.01	0.5657
Mobility scale *	5	-1.51	0.60	-0.20	<0.05	0.6022
Number of drugs taken every day	6	-0.21	0.08	-0.17	<0.01	0.6212
Living alone before nursing home placement	8	-0.69	0.49	-0.09	NS	0.6263
Difficulties in chewing	9	-0.95	0.76	-0.08	NS	0.6381

IADL – Instrumental Activities of Daily Living; GDS – Geriatric Depression Scale; B – denotes the unstandardized coefficient; SE – the standard error of B; β – the standardized coefficient beta; p – the probability value; adjusted R^2 – the adjusted squared multiple correlation; * – variables significant in stepwise backward regression of the model; NS – not significant

Discussion

The study has confirmed that malnutrition remains a common problem among older people living in nursing homes – 12% of the group studied was malnourished and 61% were at risk of malnutrition. In the study performed in all nursing homes in Helsinki one-third (29%) of the studied residents suffered for malnutrition (Mini Nutritional Assessment – MNA – score <17), and 60% were at risk (MNA 17-23.5)[17]. In these terms the study replicates also the findings of other studies [18,19].

The lower scores of Mini Nutritional Assessment were independently associated in our study with female gender, lower scores on Instrumental Activities of Daily Living scale (grater IADL dependency), higher scores on GDS (emotional disturbances), worse mobility and higher numbers of drugs taken every day since three months. In previously mentioned study, performed in Helsinki, malnutrition was associated with female gender, a longer stay in the nursing home, functional impairment, dementia, stroke, constipation and difficulties in swallowing as well as eating less than half of the offered food portion, not eating snacks and resident's weight control at long intervals, but in logistic regression analysis mainly patient--related factors predicted malnutrition (impaired functioning, swallowing difficulties, dementia, constipation) [17]. In another study performed in nursing homes the number of drugs and the mental health score were the only parameters which remained significant in the final multivariate regression model explaining the Mini Nutritional Assessment score as a dependent variable [21]. Some other researches also indicate that higher levels of nutritional disturbances in nursing homes are associated with older person ADL dependency, depression and polypharmacy [20,18].

Malnutrition in the studied group affected more frequently persons living with other people before nursing home placement, having meals in resident's room at nursing home, with the suspected dementia, personal ADL disability and with risk of pressure sores or having stroke in the past. These variables were not independently associated with the nutritional status of the older person measured as the MNA score in the regression analysis. A thorough analysis of correlations between variables revealed that these variables correlated significantly with IADL score.

As expected, the disturbances in nutrition in the present study were substantially higher in those respondents who showed to be in worse mental and physical condition. The risk of developing malnutrition in the group studied could be in a large proportion the consequence of the morbidity and frailty that has resulted in them living in long term care institution. The correlation between MNA results and age was week in the studied group. Findings of other studies were similar [21]. The frequency of malnutrition increases with age but our results suggest that it seems to be rather the function of the frailty and different chronic diseases and not the ageing per se. In case of older people living in long term care institutions a lot of disabilities and chronic diseases coexist, and health status may become more important than age in explaining malnutrition of elderly person.

Psychosocial factors such as social support and depression are related to nutritional risk in the elderly [22]. The relationship between malnutrition and depression is complex. Depression leads to reduced appetite, but, on the other hand, malnutrition may induce depression and apathy [23,24]. Insufficient dietary routines may also contribute to the risk of nutritional disturbances in nursing homes [25].

The programs that are to help prevent malnutrition in nursing home residents should encompass screening of the persons at risk, and then an intervention. Surveillance of risk factors – before full-blown malnutrition is detected – can lead to early intervention [26]. This type of intervention could decrease the risk of nutritional disturbances significantly as well as limit the negative effects of them on the morbidity and mortality of older subjects, if they should arise [27,28]. Future studies are necessary to assess to what extent these nutritional disturbances are reversible.

Conclusions

1. The study has confirmed that malnutrition remains a common problem among older people living in nursing homes.

Based on MNA one-tenth of the study subjects living in nursing home appeared to be malnourished and more then half of them appeared to be at risk of malnutrition.

2. Malnutrition is an increasing hazard especially for older women, for people with emotional disturbances and IADL dependent as well as for subjects taking greater number of drugs.

3. Introducing the periodic assessment of the nutritional status among older residents in nursing homes with simple measures such as, for example, the MNA tool, could allow for the implementation of an appropriate nutritional intervention in specific cases.

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Myeloid and lymphoid dendritic cells in the peritoneal fluid of women with ovarian cancer

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Abstract

Purpose: The aim of the study was to estimate the myeloid and lymphoid subpopulation of dendritic cells (DCs) in the peritoneal fluid (PF) of women with ovarian tumors.

Material and methods: We studied 34 patients with serous cystadenocarcinoma and 29 women with serous cystadenoma. Dendritic cells were isolated from peritoneal fluid, stained with monoclonal antibodies anti-BDCA-1 and anti-BDCA-2 and estimated using flow cytometry.

Results: Peritoneal fluid myeloid DCs constituted 0.59% of mononuclear cells in patients with ovarian cancer and 7.2% in women with serous cystadenoma. Lymphoid DCs constituted 0.39% of PF mononuclears in women with ovarian cancer and 0.07% in patients with serous cystadenoma. The percentage of lymphoid DCs was higher in patients with ovarian cancer than in women with serous cystadenoma.

The BDCA-1/BDCA-2 DCs ratio in peritoneal fluid of patients with serous cystadenoma was significantly higher in comparison to ovarian cancer.

Conclusions: Decreased BDCA-1/BDCA-2 DCs ratio in patients with ovarian cancer may favour Th2 lymphocyte differentiation and/or induction of immunological tolerance.

Key words: dendritic cells, peritoneal fluid, ovarian cancer.

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Introduction

Ovarian cancer is the fifth most common cancer in women and it is responsible for 5% deaths of females and over 50% of deaths caused by cancer of the female genital tract. Prognosis of the disease depends on several factors, including tumor margin, vascular invasion, tumor grade and stage, expression of oncogenes, and estrogen and progesterone receptors. Another possible prognostic factor are immune cells infiltrating the tumor [1].

Dendritic cells are the most potent antigen-presenting cells (APC). Two distinct DCs subpopulations of myeloid and of lymphoid origin have been described in humans. Myeloid DCs are a major subpopulation of human blood DCs which are $CD4^+$, Lin^-, $CD11c^{bright}$, $CD123^{dim}$, $CD45RO^+$ and $CD2^+$. They express myeloid markers (CD13, CD33) as well as Fc receptors (CD32, CD64, FcεRI). Myeloid DCs (DC-1) also express the BDCA-1 (CD1c) antigen which is characteristic for immature peripheral blood (PB) myeloid DCs [2,3].

Lymphoid dendritic cells (DC-2) have been described recently in human PB and lymphoid tissue. DC-2 cells express a specific BDCA-2 marker. Immunophenotyping of $BDCA\text{-}2^+$ dendritic cells characterises these cells as being $CD4^+$, Lin^-, $CD11c^-$, $CD123^{bright}$, $CD45RA^+$, $CD2^-$ and expressing neither myeloid lineage markers nor Fc receptors [3]. According to the myeloid or lymphoid origin DCs differ not only in immunophenotype but also in morphology and function. For the problem of anti-tumor immune response it could be important that myeloid DCs prime Th1 response as opposite to lymphoid DCs which prime Th2 response and tolerance [2,4].

The purpose of our study was to determine whether there are alterations of the DCs cells subsets and in the BDCA-1/ /BDCA-2 dendritic cell ratio in the peritoneal fluid between women with non-malignant and malignant ovarian tumors.

Material and methods

Of 59 women, aged 16-85 years, 34 patients were found to have FIGO stage III ovarian epithelial cancer with mean levels of Ca-125 marker 1853.97. No one women had neoadjuvant chemotherapy. Twenty nine women with benign tumors of ovaries were assigned to the reference group. The study was approved by the Lublin University School of Medicine Ethics Committee.

PF was aspirated from the anterior and posterior cul-de-sacs and taken into heparinized tubes. Mononuclear cells were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway). The cell surface antigens were determined on fresh cells at the time of a sample submission.

Isolated cells (1 x 10^6) were incubated for 20 minutes at 4°C with monoclonal antibodies (Moabs) specified against DCs surface antigens. The following monoclonal antibodies were used: anti-BDCA-1 (CD1c) FITC, anti-BDCA-2 FITC (Miltenyi Biotec) and anti-CD19 CyChrome, anti-CD123 PE (Pharmingen). Flow cytometric analysis of stained samples was performed on FacsCanto instrument (Becton Dickinson). A total of 300000 events were acquired and analysed using FacsDiva software. Cell debris and dead cells were excluded from the analysis based on scatter signals.

BDCA-1(CD1c) marker is expressed on a subpopulation of $CD19^+$ small resting B lymphocytes. The mononuclear cell analysis region was analysed for BDCA-1 (CD1c) and CD19 staining. BDCA-1 ($CD1c^+$) B cells were excluded from $CD1c^+$ peritoneal DCs by counter-staining for CD19. BDCA-1 ($CD1c^+$) $CD19^-$ cells were counted as immature myeloid DCs. Next, the mononuclear cell analysis region was analysed for BDCA-2 and CD123 antigens. BDCA-2^+CD123^+ cells are counted as lymphoid DCs (*Fig. 1*).

The results were expressed as a median and ranges. Statistical differences between groups were estimated using a standard non-parametric test (Mann-Whitney U test). P value less than 0.05 was considered as statistically significant.

Results

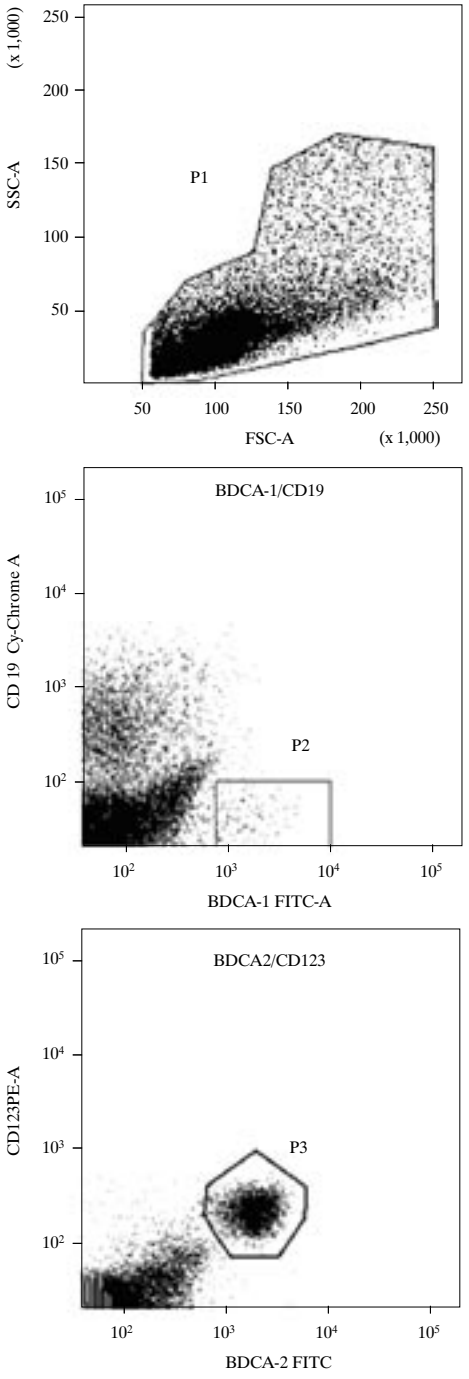
Both immature myeloid and lymphoid dendritic cells are detectable in all samples of PF of women with ovarian tumors (*Tab. 1*). Significantly higher percentages of myeloid in compared to lymphoid DCs in PF of women with serous cystadenoma were detected. There were no difference between the percentages of myeloid and lymphoid DCs in women with ovarian cancer.

The percentage of mononuclear cells that were identified as myeloid DCs was significantly higher (7.2% vs 0.59%) in women with non-malignant ovarian tumors in comparison to patients with ovarian cancer.

Lymphoid DCs constituted 0.39% of mononuclear cells in women with ovarian cancer and 0.07% in patients with serous cystadenoma. The percentage of lymphoid DCs was higher in patients with ovarian cancer than in women with serous cystadenoma (*Tab. 1*).

Significantly higher myeloid/lymphoid DCs ratio in PF of patients with non-malignant ovarian tumors (118.94) in comparison with ovarian cancer patients (1.41) was found.

Figure 1. **The identification of PF dendritic cells by flow cytometry region. The mononuclear cell analysis region (P1)applied to light scatters. The P1 gated events were analysed for BDCA-1 (CD1c) and CD19 staining and BDCA-1^+CD19^- cells were counted as immature myeloid DCs (P2). The P1 gated events were then analysed for BDCA-2 and CD123, and BDCA-2^+CD123^+ cells were counted as lymphoid DCs (P3)**



Discussion

Women with advanced ovarian cancer have often been shown to progressively develop impaired immune responses against autologous tumor cells, preceding the development of a more generalized state of immunosuppression [5]. Different

Table 1. **The percentages (% of mononuclears) of myeloid and lymphoid DCs in peritoneal fluid (PF) of patients with ovarian cancer and serous cystadenoma**

Group of patients	BDCA1$^+$/CD19$^-$ (%)			BDCA2$^+$/CD123$^+$ (%)			The BDCA-1/ BDCA-2 ratio
	Median	Min	Max	Median	Min	Max	
Cyst Adenocarcinoma	0.59	0.02	4.32	0.39	0.03	4.47	1.41
Serous Cystadenoma	7.2	0.62	24.48	0.07	0.01	0.43	118.94

mechanisms responsible for this phenomenon were described: such as generation of tumor-induced suppressor cells, alternations in T lymphocytes signal transduction, tumor induction of specific T cells apoptosis and development of peripheral tolerance to cancer's antigens [6,7].

DCs are the most potent antigen-presenting cells playing a key role in the induction of protective immune responses and maintenance of immunological memory. Their exceptional ability to instruct naïve T cells to initiate immune responses is critically beneficial for the host defence against neoplastic cells [2]. Furthermore, a large body of literature demonstrates a close relationship between the presence of DCs within various malignant tumors and prognosis. Thus, it was confirmed that DCs infiltration of solid tumors correlates with a better prognosis in head and neck tumors [8], melanoma [9], uterine cervical carcinoma [10] and ovarian cancer [1]. On the other hand it has been reported that in human suffering from cancer subpopulations of DCs are dysfunctional and consist of the phenotype immature DCs [7,11]. Several recent studies showed a significant decrease in the proportion and absolute numbers of DCs in peripheral blood [11,12]. The decrease of DCs in peripheral blood from cancer patients closely correlated with the stage and duration of the disease [11].

In our research we have made an effort to estimate the immature myeloid and lymphoid dendritic cells in PF and to investigate quantitative differences in subpopulations of DCs in women with ovarian cancer in comparison to DCs in non-malignant ovarian tumors.

We found that the percentage of myeloid DCs was significantly higher in the group of non-malignant ovarian tumor in comparison to patients with ovarian cancer. These results suggest that PF myeloid DCs population may be affected by the presence of the malignant disease and might contribute to diminished acquired immune responses observed in these women. In contrary, the percentage of lymphoid DCs was significantly higher in patients with ovarian cancer than in the reference group. This fact may be important for understanding of the mechanism of tumor immune escape, because lymphoid DCs are expected to induce tolerance rather than immunity. In study by Zou et. al. [4] it was shown that lymphoid DCs infiltrating ovarian carcinoma inhibited tumor-specific immunity by suppressing T cell activation. The investigation of Curiel et al. [13] shows comparable results in tumor ascites of women with ovarian carcinomas. They demonstrated that numerous functional lymphoid DCs accumulate in tumor ascites and inhibit antitumor immunity. The same authors found that lymphoid DCs produce high levels of the angiogenic cytokines (TNF-α and IL-8) and induce potent neovascularization in vivo. Thus, tumors may manipulate DCs distribution and alter DCs function to support tumor angiogenesis.

Other studies documented a significant dysfunction of type 1 T cell responses in tumor-bearing hosts, suggesting that tumor progression might be associated with a preferential type 2 T cell response [5]. However, factors which influence the Th2 predominance in tumor patients still remain enigmatic. The available data indicate that BDCA-1 and BDCA-2 DCs were claimed to stimulate Th1 and Th2 types of immune responses, respectively [2,3]. In our study we have demonstrated a significant accumulation of immature lymphoid DCs in the ascites of ovarian cancer patients. These cells may actively suppress the Ag-specific T cell response and thus could be involved in immunosuppression. Therefore, we concluded that PF lymphoid DCs in patients with ovarian cancer may favour Th2 lymphocyte differentiation and/or the induction of immunological tolerance which is now considerate one of the important mechanisms of tumor escape from immune system control.

The knowledge about DCs function and standardization of DCs culture conditions might represent a useful tool for cancer immunotherapy.

A further studies of the DCs function are necessary for complete understanding the influence of tumor microenvironment on DCs.

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Effect of hydralazine on CD3-ζ chain expression in Jurkat T cells

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Abstract

Purpose: Deficient CD3-ζ chain expression in T cells of patients with idiopathic SLE is associated with T cell receptor/ /CD3 complex (TCR/CD3)-mediated signaling defect. Hydralazine (HYD) inhibits expression of DNA methyltransferase 1 (DNMT1) and may cause a lupus-like disease in man.

Material and methods: To explain the HYD effect on intracellular level of CD3-ζ chain in Jurkat T leukemia cells clone E6-1, we employed the flow cytometric analysis.

Results: We observed a dose-dependent increase in cellular content of CD3-ζ chain in Jurkat T cells treated with HYD. Our results suggest that HYD may result in T cells dysfunction different from this observed in idiopathic SLE T cells.

Conclusions: This difference may partially explain distinct disease course in patients with HYD induced and idiopathic SLE.

Key words: hydralazine, T lymphocytes, cell signalling, DNA methylation.

Introduction

Systemic lupus erythomatosus (SLE) is autoimmune disease characterised by abundant production of autoantibodies. Defect in $CD4^+$ T lymphocytes signaling can be responsible for improper immune response development in patients with SLE [1].

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The T cells stimulation is initiated by binding TCR/CD3 with an antigen coupled to the major histocompatibility complex [2]. The ζ chain is component of CD3 complex and plays a major role in intracellular signaling transduction, which activate second messenger and transcription factors [2-4]. T cells stimulation induces cytokines production, increases proliferation and augmentation of effector function of T cells [2].

The defect in T cells signalling can be responsible for improper immune response development in patients with SLE [5].

It has been reported that low methylation of CpG residues in the regulatory sequences of DNA and high level of histone acetylation correlate with transcriptional activity of numerous genes [6-8]. During DNA replication, the CpG pairs of the newly biosynthesised DNA strand are methylated by DNA methyltransferase 1 (DNMT1) [6]. Expression of DNMT1 is partially regulated by extracellular signal regulated kinase pathway (ERK), and activity of this pathway is decreased in T cells from SLE patients [9]. Hydralazine (HYD) is a substance, which is able to induce a lupus-like syndrome in man. HYD inhibits ERK pathway resulting in decrease of DNMT1 expression and DNA hypomethylation [10].

Using the flowcytometric analysis, we evaluated the effect of HYD on CD3-ζ chain content in Jurkat T leukemia cells.

Material and methods

Reagents and Antibodies

HYD and digitonin were obtained from Sigma Chemical Co. (St. Louis, MO). (PE)-conjugated anti-CD3-ζ (6B10.2) mouse monoclonal antibody (MmAb) was purchased from Santa Cruz Biotechnology, Inc. USA.

Cell culture and HYD treatment

Jurkat T leukemia $CD4^+$ cells clone E6-1 were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI1640 (GibcoBRL, Grand Island, NY)

medium containing 10% heat-inactivated foetal bovine serum (FCS), 2 mM glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. Jurkat T leukemia cells were suspended at a concentration $0.5x10^6$ cells/ml in culture medium and grown for 48 h without, or in the presence of HYD in concentration of 0.5, 5.0 and 50.0 μM.

Flow cytometric analysis

After incubation, the cells were harvested and washed three times in phosphate buffered saline (PBS) supplemented with 2% FCS and 1% sodium azide (PBS/FCS).

The cells were permeabilized with digitonin 10 μg/ml, fixed with 0.25% paraformaldehyde and washed three times with PBS/FCS. The cells were then stained with PE-conjugated anti CD3-ζ MmAb, washed three times with PBS/FCS and immediately analysed on FACSCanto Flow Cytometer (Becton-Dickinson, San Jose, CA). The increase of CD3-ζ chain cellular content were calculated according to (MFx-MFo)/(MFc-MFo) x 100 formula. MF is the mean fluorescence intensity of cells, which were grown in the presence (MFx) or absence (MFc) of HYD and then stained with PE-conjugated anti CD3-ζ MmAb. Control represent fluorescence intensity of cell stained with an appropriate isotope antibody (MFo).

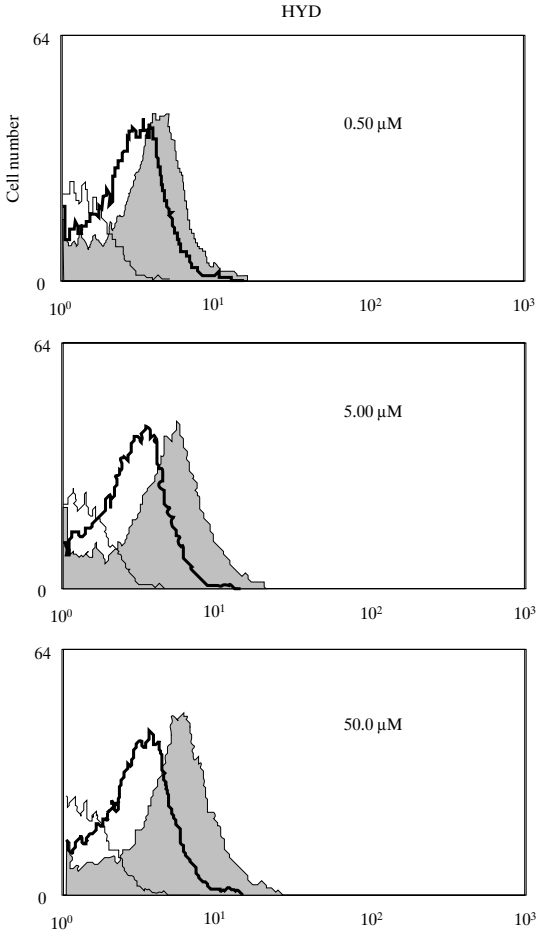
Results and discussion

In order to explain the HYD effect on intracellular level of CD3-ζ chain in Jurkat T leukemia cells, we employed the flow cytometric analysis. We observed that the HYD increased intracellular contents of CD3-ζ chain in Jurkat T cells in dose dependent manner. We have shown that the percentage increase of CD3-ζ chain formation achieves 17±3%, 70±13% and 238±40% in the presence of HYD concentration of 0.5, 5.0 and 50.0 μM, respectively (*Fig. 1*).

HYD is used to reduce the blood pressure but may also cause a lupus-like disease in man [10]. The etiology of idiopathic and HYD induced SLE is still enigmatic. Patients with idiopathic and HYD induced SLE exhibit different disease course. However, the T cells from idiopathic and drug induced SLE patients may display certain common defects at molecular level [11-13]. HYD induces biosynthesis of leukocyte function associated-1 (LFA-1) in T cells [10]. The biosynthesis elevation of LFA-1 is also observed in T cells from patients with idiopathic SLE [7]. T cells from this patients also exhibit TCR/CD3-mediated signalling aberrations, which are associated with cellular decrease of CD3-ζ transcript and protein contents.

The HYD inhibits DNMT1 expression and cause hypomethylation of regulatory DNA sequences that makes DNA template available for transcription [10,14]. The HYD causes hypomethylation of regulatory sequences of LFA-1 gene in T cells, which was also observed in the same region of DNA of SLE T cells [15]. However, we observed that HYD increased CD3-ζ protein but not transcript content (results not shown) in Jurkat leukemia $CD4^+$ T cells (*Fig. 1*). The reason for such an HYD effect on CD3-ζ chain translation is currently not known. We presume that HYD may effect on factors involved in positive control of translation or posttranslational modification of CD3-ζ chain.

Figure 1. **The representative picture of flow cytometric analysis of intracellular contents of CD3-ζ chain in Jurkat T leukemia cells incubated in the presence of HYD.**
Jurkat T leukemia cells clone E6-1 were suspended at a concentration of $0.5x10^6$ cells/ml in culture medium and were grown for 48 h either without or in the presence of HYD (0.5, 5, 50 μM). After incubation the cells were harvested, washed with PBS/FCS, permeabilized with digitonin 10 μg/ml and fixed with 0.25% paraformaldehyde. The cells were then stained with PE-conjugated anti CD3-ζ MmAb and immediately analyzed on FACSCanto Flow Cytometer (Becton-Dickinson, San Jose, CA). (—) and (–)represent expression of CD3-ζ chain in cells incubated without or with HYD. Shadow lines represents the cells stained with an appropriate isotype control antibody



Increase of CD3-ζ chain content in T cells may partially explain different disease course in patients with HYD induced and idiopathic SLE. HYD may also change other elements of TCR signaling pathway resulting in dysfunction of T cells.

The further investigation of HYD effect on expression of other signaling molecules may provide valuable information about etiology of T cells dysfunction in patients with HYD induced SLE.

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Flow cytometric analysis of CD4+ T cell receptor zeta chain deficiency in patients with systemic lupus erythematosus

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Abstract

Purpose: It has been reported that a dysfunction of T lymphocytes can be responsible for alteration in immune system in patients with systemic lupus erythematosus (SLE).

Material and methods: Using flow cytometric analysis, we determined the abnormalities of T cell receptor zeta (TCR ζ) chain contents in CD4+ T cells of SLE patients.

Results: We observed a decrease in mean fluorescence intensity of TCR ζ in CD4+ T cells of patients with SLE. The multiple analysis did not show a correlation between gender, age, disease specific manifestation, treatment, duration and TCR ζ mean fluorescence intensity in CD4+ T cells.

Conclusions: High prevalence of TCR ζ chain deficiency in CD4+ T cells confirms the significance of this signaling molecule in SLE pathogenesis.

Key words: SLE, TCR ζ, flow cytometry.

Introduction

The etiology of systemic lupus erythematosus (SLE) is still elusive. The abundant production of autoantibodies result in immune complexes formation. These immune complexes can be deposited in different tissues causing abnormal function of organs and clinical manifestation of SLE [1-3].

The T cells stimulation is triggered by direct interaction of the T cell receptor/CD3 complex (TCR/CD3) with an antigen bound to the major histocompatibility complex on antigen-presenting cells [4,5]. The TCR ζ is a component of CD3 complex and plays a pivotal role in the signaling cascade, which triggers biochemical events including second messenger and, transcription factors activation [5].

It has been reported that a dysfunction of T lymphocytes can be responsible for alteration in immune system in patients with SLE [6]. SLE T cells exhibit signaling defects, which result in enhancement of TCR/CD3 complex responses. These include disease-specific increase of intracytoplasmic calcium concentration and high-level phosphorylation of various intracellular proteins tyrosyl residue [1]. These receptor-mediated signaling aberrations are associated with deficient TCR ζ chain expression in patients with SLE [7]. The defective expression of the TCR ζ chain in SLE T cells has been determined by Western blotting technique [7].

We decided to evaluate the usefulness of flow cytometry analysis for evaluation of TCR ζ chain deficiency in peripheral CD4+ T cells of patients with SLE.

Material and methods

Thirty patients (*Tab. 1*) tested in this study were diagnosed as having SLE according to the 1982 revised criteria of American College of Rheumatology [3]. Control samples (20 women and 2 men) were obtained from age and sex matched healthy individuals. The protocol of the study was approved by the Local Ethical Committee of Karol Marcinkowski University of Medical Sciences, Poznan. Written informed consent was obtained from all participating subjects. Patients who were on prednisone were asked not to take this medication for at least 24 h before drawing the blood. All participating patients were in active stages of the disease, which were determined by Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (*Tab. 1*).

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Ficoll-Hypaque (d=1.077 g/cm^3) [8]. For flow cytometry analysis, the PBMC were incubated for 30 min

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Table 1. **Demographic, organ involvement and immunological findings of SLE patients**

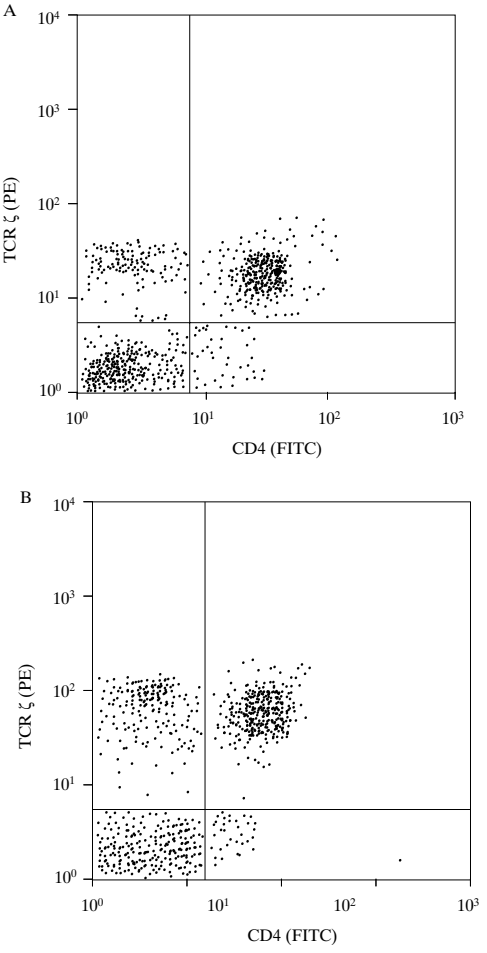
Features	
Number	30
Sex (Female/Male)	27/3
Median of age at diagnosis (years)	30 (range 19-58)
Median of disease duration (years)	6 (range 1-12)
Central nervous system*	5/30
Vascular*	3/30
Renal*	11/30
Musculoskeletal*	14/30
Serosal*	6/30
Dermal*	13/30
Immunologic*	6/30
Constitutional (fever)*	2/30
Hematologic*	10/30
Prednisone treated patients[b]	25/30
Hydroxychloroquine treated patients	6/30
No medication treaded patients	5/30
Median of SLEDAI scores	7 (range1-28)

* As defined by SLEDAI score index [6]; [b] Ten, seven, four, three and one patients were respectively receiving prednisone in dosage 10, 15, 20, 30 and 40 mg per day

with anti-CD4 Leu3A (Becton-Dickinson, Mountain View, CA) mouse monoclonal antibody (MmAb) conjugated with fluorescein isothiocyanate (FITC). After incubation, the cells were washed three times in phosphate buffered saline (PBS) supplemented with 2% FCS and 1% sodium azide (PBS/FCS). The cells were permeabilized with digitonin 10 μg/ml, fixed with 0.25% paraformaldehyde and washed three times with PBS/FCS. The cells were then stained with phycoerythrin (PE)-conjugated anti-TCR-ζ.

(6B10.2) MmAbs (Santa Cruz Biotechnology, Inc. CA) washed three times with PBS/FCS and immediately analysed in FACSCanto (Becton-Dickinson, Mountain View, CA). The TCR-ζ density on surface of cells was expressed as average of fluorescence intensity of analysed cells population (mean fluorescence intensity).

Figure 1. **Representative picture of flow cytometry analysis of TCR-ζ contents in CD4+ T cells of SLE patients (A) and healthy individuals (B).**
PBMC were incubated for 30 min with anti-CD4 Leu3A MmAb conjugated with FITC. After incubation, the cells were washed three times in PBS/FCS. The cells were permeabilized with digitonin 10 μg/ml, fixed with 0.25% paraformaldehyde and washed three times with PBS/FCS. The cells were then stained with phycoerythrin (PE)-conjugated anti-TCR-ζ (6B10.2) MmAbs washed three times with PBS/FCS and immediately analysed in FACSCanto (Becton-Dickinson, Mountain View, CA)



Results

Using flow cytometric analysis we observed a decrease in the expression of the TCR ζ chain in peripheral CD4+ T cells of SLE patients. The medians of TCR ζ mean fluorescence intensity were 44.0 (range 38.5-49.0) and 71.0 (range 48.5-74.5) in patients with SLE and healthy individuals (*Fig. 1*). The multiple analysis did not show correlation between gender, age, disease specific manifestations, treatment, duration and TCR ζ mean fluorescence intensity in CD4+ T cells. Our studies also showed the usefulness of flow cytometric analysis for determination of TCR ζ chain deficiency in CD4+ T cells. This analysis is easier and less time consuming than Western blotting technique. Using the flow cytometry analysis we confirmed the results of previous studies, which determined by Western blotting analysis the TCR ζ chain deficiency in total subset of SLE T cells [7].

Discussion

Present investigations of mechanism responsible for deficiency of TCR ζ chain in SLE T cells suggest abnormal expression of ζ chain at transcription and protein level [9,4]. Disregulation at transcription level encompasses defective expression of transcription factor Elf-1 as well as defective splicing of ζ heteronuclear RNA [8]. Recent investigations employing histone deacetylase inhibitor Trichostatin A indicates the possibility that abnormal chromatin structure can be also responsible for the decrease in ζ chain content in SLE T cells [10-12].

The TCR ζ can be replaced functionally by FcR gamma chain in SLE T cells [13]. The FcR gamma is a member of TCR ζ chain protein family and is also a component of the high-affinity IgE receptor [14]. The FcR gamma may mediate abnormal signal transduction through Syk kinase, which is more potent

than ZAP-70 kinase cooperating with TCR ζ [5]. The decrease of TCR ζ and increase of FcR gamma content in SLE T cells can be responsible for TCR/CD3-mediated signaling, which differs from the normal T cells [5]. To skew of TCR ζ and FcR gamma expressions, drugs or gene therapy can be used, resulting in correction of the defective signal transduction in SLE T cells [8].

High prevalence of TCR ζ chain deficiency in $CD4^+$ T cells of SLE patients indicates the significance of this signaling molecule in SLE pathogenesis. However, the effect of TCR ζ chain deficiency on mechanism of cytoplasmic proteins hyperphosphorylation and increase in SLE T cells profile calcium response, requires further investigation.

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Photodynamic diagnosis (PDD) using 5-aminolevulinic acid-supplemented cultures of human endometrial epithelial cells

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Abstract

Purpose: The studies were aimed at monitoring 5-aminolevulinic acid (5-ALA)-dependent accumulation of endogenous protoporphyrin IX (PpIX) in epithelial cells originating from normal endometrium or endometriotic foci, as related to steroid treatment.

Material and methods: Epithelial cells were cultured in presence of estradiol-17 beta (E2) and progesterone (P) in concentrations typical for the follicular stage (E2 alone, 220 pg/ml) or the luteal stage (E2 100 pg/ml and P 2 ng/ml) or in presence of progesterone alone (2 ng/ml) for a period of 24, 48 or 72 h. Effect of 5-ALA concentration on the accumulation of PpIX was defined in the cells incubated with 2.0 mmol/l 5-ALA for 2 h. PpIX fluorescence was detected using a confocal microscope.

Results: After hormonal stimulation, intensity of PpIX-specific fluorescence was only slightly increased in epithelial cells originating from normal endometrium. Cultures of epithelial cells from endometriosis foci showed higher concentration of PpIX than did the cells originating from normal endometrium. The highest peak of PpIX fluorescence was noted in epithelial endometriotic cells after 48h incubation with progesterone.

Conclusions: The data on PpIX accumulation in epithelial cells in the presence of estradiol-17 beta or progesterone may provide indications as to the menstrual cycle phase(s) in which photodynamic therapy for endometriosis should be performed. It is concluded that hormonal condition of female body must be taken into account for diagnosis and treatment of endometriosis.

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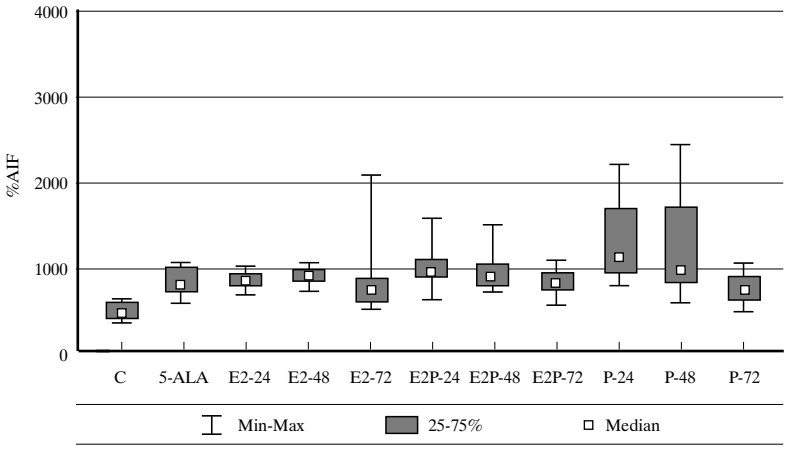
Key words: endometriosis, 5-aminolevulinic acid, protoporphyrin IX, estradiol-17 beta, progesterone.

Introduction

Endometriosis is a disease of endometrial glands and stromal cells, developing out of the uterine cavity. It is appraised to affect 3-10% women in the generative age but among infertile women and in women with pain in the pelvis the incidence is supposed to reach 20-90% [1]. In clinical practice the only reliable way to diagnose endometriosis is to visualize its typical lesions in the course of laparoscopy or laparotomy and to confirm the diagnosis by histopathology. In recent years a photodynamic technique has been introduced to clinical practice, both for diagnostic and therapeutic purposes. It uses fluorescent drugs that concentrate preferentially in tumours and other hyperproliferative tissues. At present, among substances applied in the photodynamic approach particular attention is focused on 5-aminolevulinic acid (5-ALA), the presence of which represents a physiological requirement for heme production in cells protoporphyrin IX (PpIX) represents one of the compounds which arise during heme biosynthesis and is used in photodynamic therapy (PDT) as a photosensitizer [2].

Several studies have demonstrated the capacity of endometrium to accumulate more ALA-dependent PpIX as compared to other tissues [3-10]. The obtained data allow us to suggest that this compound can be used for diagnosis and treatment of endometriosis, when hormonal condition of female body is taken into account. Recognition of PpIX accumulation in isolated cells of endometrial epithelium (from uterine cavity) and of endometriotic foci, as affected by estrogen and progesterone, may be practical significance for definition of requirements of photodynamic diagnosis of endometriosis and its therapy.

Figure 1. **Alterations in PpIX content in epithelial cells isolated from normal endometrium during incubation with steroids and 5-ALA. C: control cells, 5-ALA: 2 h incubation with 2 mmol/l 5-aminolevulinic acid; E2-24/48/72: time of preincubation with estradiol subsequently 24, 48 or 72 h and 5-ALA-2 h; E2P-24/48/72: time of preincubation with estradiol and progesterone for 24, 48 or 72 h and 5-ALA-2 h; P-24/48/72: time of preincubation with progesterone for 24, 48 or 72 h and 5-ALA-2 h**



Material and methods

The studies were performed on human primary epithelial cells, originating from normal uterine cavity or from genital or and ovarian endometriotic foci in five patients. The cells were isolated and cultured as described by Ryan et al., with some modifications [11]. Immediately after biopsy, tissue material was stored in F12 fluid (Sigma) supplemented with antibiotics: 10 μg amphotericin/ml (Sigma) and 0.2 mg gentamycin/ml (Sigma). The material was dissected into fragments of 1 mm^2 in area and subsequently transferred into F12 medium containing collagenase (0.25%, GIBCO, 189 U/mg). The tissue pieces were subjected to shaking in 37°C water bath for 2 h. Cells of the glands were separated from stromal and blood cells by filtration through sieves of 38 to 105 μm pore diameters. Glands were recovered from the sieves and placed into F12 medium with trypsin (0.25%, 15 minutes). The epithelial cells were incubated in F12 growth medium, containing 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mmol/l L-glutamine and 10% foetal calf serum (FCS). All the subsequent experiments on the cells were conducted following 4 days of culture.

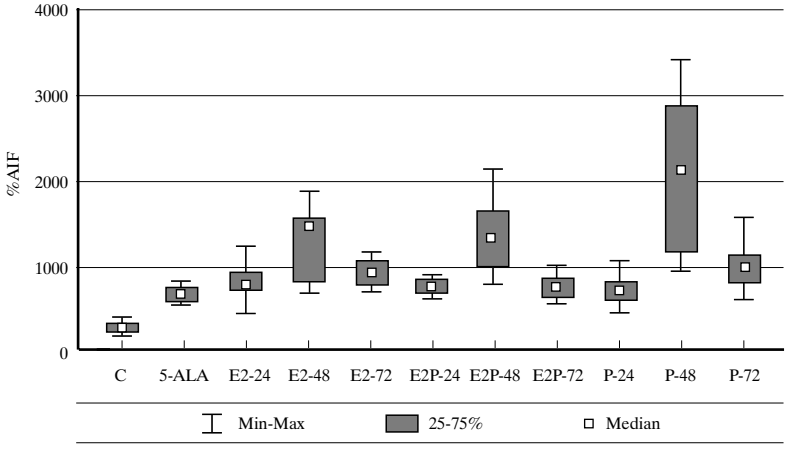
Cultures of epithelial cells, isolated from normal endometrium and endometriosis, were conducted in presence of estradiol-17 beta (E_2) and progesterone (P) in concentrations typical of the follicular stage (E_2 alone, 220 pg/ml) or the luteal stage (E_2 100 pg/ml and P 2 ng/ml) for a period of 24, 48 or 72 h (the hormone doses were selected to correspond to their blood levels during normal menstrual cycles in women) [12]. Effect of 5-aminolevulinic acid (5-ALA) concentration on accumulation of protoporphyrin IX (PpIX) in cells was defined in cells following their incubation with 2.0 mmol 5-ALA per l ml culture medium for a period of 2 h (5-ALA concentration and duration of incubation were selected on the basis of published data and our preliminary results) [2,4,13]. Following that time, PpIX content in cells was evaluated using a confocal microscope (LSM 510, Zeiss). The estimations took advantage of PpIX-exciting laser beam of 458 nm wavelength (argon laser, HFT 458), while the emitted light was analysed using 585 nm filter (LP 585). PpIX content was evaluated using the CytFlu 1.2 software and expressed as a product of an average intensity of fluorescence and percentage surface bearing area over the level of background (%AIF). In each experiment, cells of control group were evaluated, incubated in the same way but in the culture medium devoid of 5-ALA. Statistical evaluation of the obtained results involved the nonparametric U-Mann Whitney's test, performed using Statistica ver. 5 software. P value <0.05 was considered to represent threshold of significance.

Results

Results of PpIX-specific fluorescence estimation in a confocal microscope following preincubation of normal epithelial cells with steroids and incubation with 5-ALA are presented in *Fig. 1*. Protoporphyrin IX content, following 2h incubation with 2 mmol/l ALA without steroid treatment, was 675 %AIF. After hormonal stimulation with estradiol-17 beta and estradiol-17 beta plus progesterone, intensity of PpIX-specific fluorescence was only slightly increased in epithelial cells originating from normal endometrium. The maximum degree of PpIX accumulation (1023% AIF) was noted after 24 h preincubation with progesterone (*Fig. 1*).

A separate cycle of experiments was devoted to alterations in PpIX content in epithelial cells isolated from endometriotic foci, which were preincubated with steroids and, then, transferred to the 5-ALA-containing medium. The obtained results are illustrated in *Fig. 2*. The cultured epithelial cells from endometriotic foci showed higher concentration of PpIX than the cells originating from normal endometrium, especially in the case of 48 h preincubation with hormones. Following the 48 h/estradiol-17 beta treatment of the epithelial cells, intensity of the PpIX-specific fluorescence significantly increased

Figure 2. **Alterations in PpIX content in epithelial cells isolated from endometriotic foci during incubation with steroids and 5-ALA. C: control cells, 5-ALA: 2 h incubation with 2 mmol/l 5-aminolevulinic acid; E2-24/48/72: time of preincubation with estradiol subsequently 24, 48 or 72 h and 5-ALA-2 h; E2P-24/48/72: time of preincubation with estradiol and progesterone for 24, 48 or 72 h and 5-ALA-2 h; P-24/48/72: time of preincubation with progesterone for 24, 48 or 72 h and 5-ALA-2 h**



($p<0.05$) but was followed by a significant decrease ($p<0.05$) after 72h (*Fig. 2*). Likewise, in the case of estrogen plus progesterone treatment a significant increase ($p<0.05$) in cellular PpIX content was noted after 48h. However, the highest peak of protoporphyrin IX fluorescence (2115% AIF) was observed after progesterone treatment in 48th h of the experiment (*Fig. 2*). PpIX-specific fluorescence in cells of the control group did not significantly change in the course of the entire experiment and never exceeded the value of 240%AIF.

Discussion

For a properly conducted photodynamic therapy, an appropriate photodynamic diagnosis is pre-required. The finding that 5-aminolevulinic acid (5-ALA), which basically is not a photosensitizer itself, induces accumulation in cells (particularly in tumour cells) of endogenous protoporphyrin IX has proved especially significant. 5-ALA is a naturally occurring metabolite in the heme biosynthesis pathway. It is synthesized in the mitochondrial matrix from glycine and succinyl-CoA under the effect of 5-ALA-synthase. Subsequently, ALA finds its way to the cytoplasm, in which in the presence of 5-ALA-dehydratase (ALA-D) it becomes condensed to porphobilinogen. As the result of subsequent reactions (deamination, decarboxylation and oxidation), catalysed by appropriate enzymes (porphobilinogen-deaminase, uroporphyrinogen-decarboxylase, coproporphyrinogen-oxidase and protoporphyrinogen-oxidase) PpIX is formed, from which, following addition of iron (mitochondrial ferrochelatase), heme is produced [14]. In cases of augmented heme production, activity of 5-ALA-synthase is inhibited due to a negative feedback [15]. Excess exogenous 5-ALA circumvents feedback inhibition, leading to accumulation of highly fluorescent and photosensitizing porphyrins, mainly protoporhyrin IX. Accumulation of PpIX in cells under effect of ALA used to be evaluated by analysis of fluorescence intensity. In our studies, the measurement of fluorescence intensity was performed using confocal microscope. The results have demonstrated that incubation in media containing steroids (estradiol-17 beta, progesterone) and 5-aminolevulinic acid induce accumulation of PpIX in the isolated epithelial normal cells and epithelial cells isolated from endometriotic foci. The evident decrease in the fluorescence intensity was noted always following 72 h incubation with steroids. The decrease in PpIX content in the studied cells might have reflected efflux of the compound or increased activity of ferrochelatase, which catalyses binding of PpIX with iron. Moreover, application of progesterone for 48 h and ALA for 2h resulted in a twofold increase in PpIX-related fluorescence in epithelial cells isolated from endometriotic foci only. At the moment, the mechanism of this phenomenon remains difficult to interpret but one should keep in mind the potential for induction of enzymes responsible for ALA synthesis and possibly lower efflux of the compound from the cells. It is certainly important for future investigations; it seems that in certain conditions the cell may preferentially switch to an endogenous mechanism under progesterone stimulation, inducing a higher intracellular accumulation of the sensitizer.

The data on PpIX accumulation in endometrial cells as related to presence of estradiol-17 beta or progesterone in incubation medium may provide indications as to the menstrual cycle phase(s) in which PDD and/or PDT for endometriosis treatment should be performed. It is concluded that for diagnosis and treatment of endometriosis hormonal condition of female body must be taken into account.

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Immature reticulocyte fraction (IRF) – an universal marker of hemopoiesis in children with cancer?

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Abstract

Purpose: Anemia is one of the most frequent side effects of anticancer treatment, it is also caused by disease itself. Reliable laboratory tests indicating hematological recovery after chemo- and radiotherapy are needed. Effective erythropoiesis can be monitored by quantitative measurement of reticulocytes. The amount of RNA in these cells can be assessed with flow cytometry and divided into low- (LFR), middle- (MFR) and high-fluorescence reticulocytes (HFR). This distribution is correlated with their maturation.

Material and methods: The aim of our study was to find the most sensitive indicator of anaemia among reticulocyte subpopulations assesed by flow cytometry in children with cancer. 46 children with different neoplasmatic diseases were enrolled into the study.

Results: 1) we did not find any differences between control and examined group at the time of diagnosis except for IRF, which was higher in examined group ($p=0.001$); 2) IRF was lower already 2-4 days after end of chemotherapy ($p=0.03$), and rised before next regimen ($p=0.0006$).

Conclusions: In conclusion we showed that IRF is not only the first sign of hematologic recovery but also very strong indicator of postchemotherapy aplasia in children with cancer and may serve as a additional parameter of bone marrow function in clinical studies.

Key words: anaemia, reticulocytes, IRF, flow cytometry, cancer, children.

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Introduction

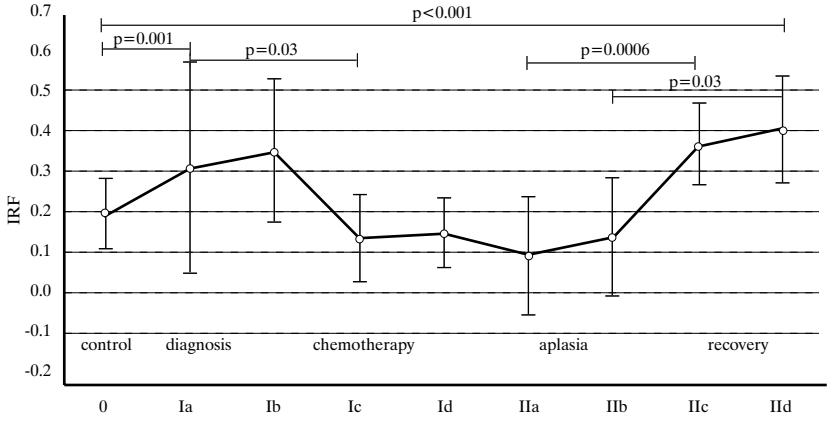
Anemia is one of the most frequent side effects of anticancer treatment, it is also caused by disease itself. Reliable laboratory tests indicating hematological recovery after chemo- and radiotherapy are needed. Effective erythropoiesis can be monitored by quantitative measurement of reticulocytes [1,2]. The amount of RNA in these cells can be assessed with flow cytometry and divided into low- (LFR), middle- (MFR) and high-fluorescence reticulocytes (HFR). This distribution is correlated with their maturation. HFR fraction represents the most immature reticulocytes. The immature reticulocyte fraction (IRF) is the sum of HFR and MFR fractions. The usefulness of flow cytometric analysis of reticulocytes, as a predictor of engraftment in autologous bone marrow transplantation and for granulocyte recovery after polychemotherapy in leukaemic patients was reported in many studies. Earlier studies suggested HFR as a strongest predictor of hematological recovery, others – IRF. The aim of our study was to find the most sensitive indicator of anaemia among reticulocyte subpopulations assesed by flow cytometry in children with cancer.

Material and methods

46 children with different neoplasmatic diseases were enrolled into the study. Children were suffering from leukaemias, lymphomas and solid tumors. The control group consisted of 40 children subjected to Department of Pediatric Surgery for minor operations with no sign of anaemia.

Peripheral blood was taken according to physicans' orders for blood count. Resting material was used for flow cytometric assessment of reticulocytes. In total over 500 specimens were assessed. Obtained results were divided into 9 groups: control (0), time of diagnosis (Ia), 1st day after chemotherapy (Ib), 2-4 days after chemotherapy (Ic), 5-9 days after chemotherapy (Id), children admitted to the hospital in aplasia: at the beginning of hospitalisation (IIa) – WBC (white blood cells)

Figure 1. **IRF in control and examined group in consecutive stages of therapy**



<1.0 x 10^3/μl, nadir – deep aplasia (IIb) – WBC<0.5 x 10^3/μl, neutrophils <0.2 x 10^3/μl, at the beginning of recovery (IIc) – WBC>1.0 x 10^3/μl, before next chemotherapy (IId).

Reticulocytes and their subpopulations were automatically analysed by flow cytometry as described earlier [3]. Samples of venous blood containing EDTA were analysed within 4 hours using Epics XL (Coulter flow cytometer). Preparation procedure included staining with thiazole orange. Data were processed using Excel' 97 and Statistica 6.0. Results are showed as mean and percentiles. Significance levels were calculated according to the nonparametric Mann-Whitney U-test (difference between the control group – 0 and consecutive stages of therapy Ia-IId) and the Spearman correlation coefficient (correlation between all assessed parameters). Level of $p<0.05$ was regarded as significant.

Results

1) We did not find any differences between control and examined group at the time of diagnosis except for IRF, which was higher in examined group ($p=0.001$).

2) Total percentage of reticulocytes was lower in aplasia (IIa) comparing to the time of diagnosis (Ia) ($p=0.02$), but not directly after chemotherapy (Ib, Ic) and rised before next regimen (IId) ($p=0.03$); very similar tendency was observed in reticulocyte count, HFR count, MFR, LFR count and percentages;

3) We found the differences in IRF in following groups (*Fig. 1*): control group vs time of diagnosis in examined group ($p=0.001$), IRF was lower already 2-4 days after the end of chemotherapy ($p=0.03$), and rised before next regimen ($p=0.0006$);

4) We noted many correlations between all assessed parameters but here we mention only strong ones (positive, $r>0.5$, $p<0.00001$):

- between Hb or E and: number of reticulocytes, number of LFR,
- between WBC and: percentage and number of reticulocytes, MFR, LFR and IRF
- between PLT and: percentage and number of reticulocytes, MFR, LFR, IRF.

Discussion

It is known from many years that number of reticulocytes in the peripheral blood corresponds with bone marrow activity [4]. Our results concerning IRF are similar to those obtained by Spanish Multicentric Study Group for Hematopoietic Recovery: a rising IRF was the first sign of hematopoietic recovery [5]. Kabata et al. also found that HFR + MFR increase was predictive for hematological recovery but limited to bone marrow transplantation procedures [6]. In authors' opinion regeneration of reticulocytes was observed after leucocyte recovery. Torres et al., used flow cytometry to assess same parameters as in our study, but in the group of allo- and autologous transplanted patients. The authors observed earlier increase in reticulocyte parameters than rise in neutrophil count [7]. They conclude that any determined reticulocyte parameter can reliably measure this fraction, but the most useful are mean fluorescence index (MFI) and mean reticulocyte volume (MRV). On the other hand in Kuse's et al. opinion MFR (middle fluorescence fraction) and HFR may serve as indicators of bone marrow recovery after chemotherapy [8]. George et al. found that HFR preceded neutrophil recovery, so in their opinion HFR can be used as an earlier indicator of engraftment after stem cell transplantation [9].

Buttarello et al. provided a very interesting comparison in IRF measurement with 5 different automated counters [1]. In their opinion even with slight differences, the fluorescence-based methods seem to be more robust than other methods for IRF measurement.

Flow cytometry is considered as an expensive and sometimes time consuming laboratory method but differentiation of reticulocytes does not require monoclonal antibodies and assessment takes about 1.5 hours (including 1h of incubation). It is also thruth that reticulocyte count is stable after storage for 48 or even 72 hours, but IRF parameters are stable only for 8 hours [10,11].

The monitoring of bone marrow regeneration after chemo- and radiotherapy is also important because of a high risk of infection, which increases with time of aplasia [6]. We have earlier showed a weak correlation between IRF and other parameters of hemopoiesis, same results were obtained in this experiment (no correlation between E or Hb and IRF), so we suggest its independent role as a marker of erythropoietic activity [3]. In conclusion we demonstrate that IRF is not only the first sign of hematologic recovery but also very strong indicator of postchemotherapy aplasia in children with cancer and may serve as a additional parameter of bone marrow function in clinical studies.

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Protecting the peritoneal membrane in dialyzed patients

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Abstract

This review paper describes methods of protecting the peritoneal membrane in uremic patients chronically treated with peritoneal dialysis. Possible interventions involved in protection of the peritoneum aim at reducing peritoneal exposure to glucose, glucose degradation products and lactate; preventing or diminishing harmful effects of dialysis solutions; decreasing infection rate, especially peritonitis, and its consequences. Techniques reducing peritoneal exposure to bioincompatible solutions include peritoneal resting, replacing some glucose exchanges with amino acid-based, icodextrin-based or glycerol-based dialysis solution, using bicarbonate or pyruvate as a buffer, and administering solutions with low content of glucose degradation products. Preventing or diminishing harmful effects of dialysis solutions includes interventions with drugs, especially those given intraperitoneally. Decreasing local and systemic infection rate is also very or even the most important in maintaining relatively unchanged peritoneal membrane histology and function.

Key words: peritoneal membrane, dialysis solutions, peritoneal resting, drugs, infections.

Long-term peritoneal dialysis (PD) usually leads to peritoneal membrane failure. Main factors, important in pathogenesis of deterioration of the peritoneum, include continuous exposure to bioincompatible dialysis solutions and high peritonitis rate. Having in mind factors responsible for the peritoneal membrane failure, possible interventions in its protection aim in PD patients at: 1) reducing peritoneal exposure to glucose, glucose degradation products (GDPs), and lactate; 2) preventing or diminishing harmful effects of dialysis solutions; 3) decreasing infection rate, especially peritonitis occurrence and its harmful consequences.

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Reducing peritoneal exposure to bioincompatible solutions

Techniques include peritoneal resting, replacing some glucose exchanges with amino acid-based (AA-DS), icodextrin-based (PG-DS) or glycerol-based dialysis solution, using bicarbonate or pyruvate as a buffer, and administering solutions that have low content of GDPs [1].

Temporary discontinuation of continuous ambulatory peritoneal dialysis (CAPD) for 4 weeks in patients who developed a reduction in ultrafiltration capacity has been reported to lower mass transfer area coefficients (MTACs) of urea and creatinine and to increase ultrafiltration [2,3]. The effect lasted for up 12 months. More recent studies showed that peritoneal resting was especially effective when applied early after the detection of ultrafiltration failure [4] and with heparinized lavage [5]. Peritoneal resting has been associated with both an increase [6] and a decrease of dialysate glycoprotein cancer antigen 125 (CA125) concentrations [7]. In the other study [8], rats exposed to dialysis fluid for 5 weeks showed a severe angiogenesis in various peritoneal tissues, a profound fibrosis in the parietal peritoneum, a higher number of mast cells and milky spots in the omentum and severe damage to the mesothelial cell layer covering the peritoneum. The 12 weeks peritoneal rest resulted in a significant reduction in blood flow in visceral but not in parietal peritoneum, a reduced degree of fibrosis, normalization of increased mast cell density and recovered mesothelial cell layer.

AA-DS is more biocompatible than glucose-based solutions. Recently it was proven in the rat model of peritoneal dialysis [9]. Daily exposure to glucose-based solution for 5 weeks resulted

in a significant increase in the number of rolling leukocytes in mesenteric venules, whereas instillation of AA-DS did not change the level of leukocyte rolling. Glucose-based solution evoked a significantly higher number of milky spots in the omentum, whereas this response was significantly reduced in animals exposed to AA-DS. These data indicate reduced immune activation with the use of AA-DS. Quantitative morphometric evaluation showed less angiogenesis in the parietal peritoneum after treatment with AA-DS compared to glucose-based solution. Instillation of AA-DS resulted in approximately 50% reduction of fibrosis in the mesentery and approximately 25% reduction in the parietal peritoneum compared to glucose-based solution. As evidenced by electron microscopy, glucose-based solution damaged the mesothelial cell layer, whereas mesothelium was intact after AA-DS treatment.

PG-DS is mentioned as more biocompatible than glucose-based solutions. However, the recent study by Gotloib et al. [10] showed that both osmotic agents, 4.25% glucose and 7.5% icodextrin, substantially restrain the normal process of mesothelial cell repopulation and induce repair by means of connective tissue. The underlying mechanism is most likely sustained oxidative stress.

The 15 new peritoneal dialysis patients were randomized to treatment with either glucose-based or glycerol-based dialysis solutions [11]. No difference between the two groups was found after 1 and 3 months with regard to peritoneal transport kinetics, but dialysate CA-125 concentration was significantly higher in the glycerol-treated patients than in the glucose-treated ones.

Lactate itself exerts harmful effect on human peritoneal mesothelial cells (HPMC) viability. It was shown that 3,4-dideoxyglucosone-3-ene (3,4-DGE) or acidity alone inder the absence of lactate do not decrease HPMC viability. However, combination of acidity and 3,4-DGE markedly decreases viability of HPMC under the existence of lactate [12]. Lactate concentration is the major determinant of polyol pathway activation and sorbitol accumulation in HPMC. Reduction of lactate concentrations might help to limit the negative impact of dialysis solutions on peritoneal membrane and promote its long-term survival [13].

Pyruvate has induced less cytotoxicity to peritoneal macrophages and mesothelial cells than did lactate [14]. That finding can be attributed partly to the lower pH of pyruvate (which makes it a weaker buffer), but also to the ability of pyruvate to scavenge oxygen radicals [15]. Pyruvate also causes less stimulation of intracellular degradation of glucose in the sorbitol pathway [16]. Lactate increases the intracellular $NADH/NAD^+$ ratio due to inhibition of NAD^+ regeneration. A high $NADH/NAD^+$ ratio is also called pseudohypoxia [17] and is likely to stimulate the formation of vascular endothelial growth factor (VEGF) [18]. Of many potential mediators produced by mesothelial cells, VEGF was more important than IL-6 in determination of peritoneal solute transport rates in newly started nondiabetic peritoneal dialysis patients [19].

All dialysis solutions not containing glucose have advantage as not stimulating formation of advanced glycation end products (AGEs), which are well known contributors to the peritoneal membrane failure.

Heat sterilization of glucose-based peritoneal solutions increases a variety of GDPs, which directly cause cellular injury in fibroblasts, mesothelial cells and mononuclear cells. Some GDPs, like methylglyoxal, may additionally facilitate the generation of AGEs, causing ultrafiltration failure. Increase in temperature to 37°C during storage for one day (applicable in tropical countries) has minor effect on 3,4-DGE formation. Storage in temperature of 60°C even for one day significantly enhances 3,4-DGE content in dialysis fluid [20].

Exposure to dialysis solutions with neutral pH and reduced GDPs content has resulted in an increase in the effluent concentration of CA-125 [21-27], in a decrease in dialysate concentration of hyaluronan, irrespective of the buffer used [21,22,27,28], in better preservation of cobblestone-shaped mesothelial cells due to protective effect on their fibroblastoid transition [23], and less formation of AGEs [29,30].

It is commonly accepted that CA-125 levels in dialysate reflect mesothelial cell mass. Therefore, higher CA-125 concentrations indicate better preservation of the peritoneal mesothelium. When two groups of new CAPD patients (one treated with low GDP solution, second treated with high GDP solution) were compared, the low GDP group, using Balance Fresenius Medical Care (Germany), showed higher dialysate CA-125 levels during one year CAPD follow-up (55.4 ± 24.8 vs 8.8 ± 1.7 U/ml at the 1st month – $p=0.000$, 56.7 ± 28.1 vs 22.1 ± 11.5 U/ml at the 6th month – $p=0.000$, 54.2 ± 28.2 vs 24.6 ± 16.5 U/ml at the 12th month – $p=0.000$) [23]. In *ex vivo* studies, dialysate from patients treated with low GDP solution supported growth of mesothelial cells better than that obtained from the same patients on standard dialysis fluid [31]. Additionally, *in vitro* remesothelialization occurred without delay in the presence of low GDP solution but was markedly retarded by standard solution [32]. These facts, taken together, indicate that higher concentration of CA-125 observed with low-GDP solution, may reflect less harmful effects of this solution on mesothelium as compared to conventional fluid.

Glycosaminoglycan (hyaluronan) is a high molecular weight mucopolysaccharide composed of repeating dimmers of N-acetylglucosamine and glucuronic acid. Mesothelial wound healing is associated with local synthesis of hyaluronic acid, therefore lower concentration of hyaluronan in dialysate may indicate less need for remesothelialisation occurring when low-GDP solutions are used instead of standard fluid [21,22,26,28]. The rat studies seem to confirm this hypothesis [33].

The influence of low-GDP solution on chronic peritoneal inflammatory state is not clear. A decrease in dialysate concentration of IL-6 was shown, but simultaneously no influence on dialysate CRP level was observed [24].

Formation of AGEs *in vitro* [29] and in the rat model of peritoneal dialysis [30] occurs faster in the presence of standard dialysis fluid compared to low-GDP solution. The influence of low-GDP fluid on expression of VEGF and microvascular proliferation in the rat is controversial [30,34]. No significant changes in dialysate VEGF in CAPD patients were observed with the use of low-GDP solution [26,27].

Do et al. [23] have introduced scoring system for description of morphology of human peritoneal mesothelial cells: score 1=cobblestone-shaped cells, score 2=mixed, score 3=fibroblas-

toid cell dominant. New CAPD patients treated with low-GDP solution as compared to those using high-GDP solution revealed lower cell scores at the 1st, 6th and 12th months (1.22, 1.22 and 1.56 vs 1.61, 1.75 and 2.14; $p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively), and the significantly lower number of fibroblast dominant cultures at the 12th month (12.5% vs 50% patients, $p<0.05$) [23]. Human peritoneal mesothelial cells can be stained with both cytokeratin and vimentin, whereas typical fibroblasts can be stained with vimentin but not cytokeratin. Do et al. [23] demonstrated that both cobblestone-shaped mesothelial cells and fibroblastoid cells were positively stained with cytokeratin and vimentin. This indicates that fibroblastoid cells originated from epithelium, most likely in a transition from peritoneal mesothelial cells under GDP stress, although they looked like typical fibroblasts in morphology [23]. Selgas et al. [35] suggest that transdifferentiated mesothelial cells are main source of VEGF in PD patients and that an epithelial-to-mesenchymal transition of mesothelial cells is a mechanism responsible for high peritoneal solute transport rate. This transition might be the initiating lesion associated with high transport rate, independent on time on PD [36].

A superior survival was found in patients treated with a neutral pH, low-GDPs solution (Balance, Fresenius Medical Care, Germany) compared to those treated with the conventional fluid. Balance gave mortality rates of 12.2 deaths per 100 patient-years compared with 18.3 deaths per 100 patients-years for the conventional solution. On the other hand, there were no differences between the two groups for technique survival, peritonitis-free survival, or peritonitis rates [37].

Treatment of CAPD patients with combination of AA-DS, PG-DS and bicarbonate/lactate-buffered glucose-based solution for 30 weeks resulted in higher CA-125 dialysate concentration compared to standard fluid [38]. However, this low-glucose and low-GDPs regimen was not able to prevent the decrease of dialysate CA-125 level, observed after 6 weeks of dialysis follow-up. It indicates that advanced studies should be continued to improve biocompatibility of peritoneal solutions.

Preventing or diminishing harmful effects of dialysis solutions

Interventions with drugs for the preservation of the peritoneum have been studied, but such interventions have never been applied for a large scale. Drug therapy is still experimental – and to some extent disappointing [1].

Phosphatidylcholine, given intraperitoneally during CAPD, increased ultrafiltration in patients with ultrafiltration failure and with normal ultrafiltration [39,40-42]. The most likely mechanism is an effect on lymphatic absorption of fluids [43], either by direct uptake in the subdiaphragmatic lymphatics [44] or by an effect on the glycocalyx that inhibits transmesothelial transport. Phosphatidylcholine has never been employed in day-to-day clinical practice because it is extremely difficult to dissolve in dialysis solution and oral administration is not effective [45].

Intraperitoneal hyaluronan effects were examined in peritoneal dialysis patients [45,47] and in rats [48,49]. In peritoneal dialysis patients, solute (sodium, urea, creatinine, albumin, glucose) clearances, dialysate to plasma ratios and MTACs were similar with or without hyaluronan [47]. In rats, clearance of urea was higher with hyaluronan [49]. In other studies, intraperitoneal administration of glycosaminoglycan in CAPD patients was associated with reduced peritoneal protein loss [46]. In some studies hyaluronan decreased peritoneal fluid absorption [49,50] or at least net ultrafiltration tended to be slightly higher during treatment with solution containing hyaluronan compared to control treatment [47]. Recently, Flessner et al. [51] concluded that the hyaluronan concentration in the visceral peritoneal interstitium does not significantly contribute to the barrier for water flow to or from the visceral space surrounding the peritoneal cavity. Hyaluronan also revealed protective effect against peritoneal injury during repeated exposure to hypertonic dialysis solutions or 0.9% saline in rats [48,52,53]. Explanation of this finding includes suppression of the release of active oxygen from peritoneal macrophages by hyaluronan [54], which also acts as a free-radical scavenger [55].

In rats exposed to dialysis fluid supplemented with N-acetylglucosamine, peritoneal permeability to creatinine and proteins was reduced when compared to animals dialyzed with glucose solution. This effect was related to accumulation of glycosaminoglycans in the peritoneal interstitium [56,57]. Synthesis of hyaluronan by mesothelial cells was significantly increased in the presence of N-acetylglucosamine [58]. Tissue content of hyaluronic acid was increased in rats receiving N-acetylglucosamine intraperitoneally as compared to animals treated with glucose or mannitol based dialysis solutions. However, submesothelial thickness showed an increase in all rat groups [59].

In rats low molecular weight heparin – dalteparin – improved peritoneal ultrafiltration acutely due to reductions in peritoneal transport of small solutes. It is speculated that this effect may be related to the anti-inflammatory effects of dalteparin, reducing the vasodilatation normally occurring at the beginning of peritoneal dialysis dwells [60]. Using intraperitoneal dalteparin in long-term peritoneal dialysis patients, an increase in the peritoneal restriction coefficient to macromolecules was found [61]. Recent studies [62] showed that an increase in ultrafiltration caused by low molecular weight heparin was associated with inhibition of formation of thrombin and blockade of C5a activity.

Peritoneal fibrosis, and particularly peritoneal sclerosis, constitutes some of the most disastrous complications of peritoneal dialysis [63]. In the view of the good results obtained with tamoxifen for the treatment of retroperitoneal fibrosis, in 1992 Diaz-Buxo et al. [64] suggested for the first time its use in peritoneal dialysis patients. Tamoxifen inhibits protein kinase C, a mediator of cellular proliferation, and possibly inhibits other growth factors (epidermal growth factor and calmodulin). Nine of 23 patients, diagnosed with peritoneal sclerosis, were treated with tamoxifen (20 mg BID for a period of 14.5 ± 7 months) and 14 patients served as controls [65]. None treated patient did not develop encapsulating peritoneal sclerosis (EPS) and overall mortality rate was 22%, whereas in non-treated group 4 patients developed EPS and 71% died ($p=0.03$).

The rat studies indicate that angiotensin II blockade may be a potential means of preventing fibrosis of the peritoneal mem-

brane [66,67]. Duman et al. [66,67] found that, in rats receiving high glucose dialysis solutions for 4 weeks, simultaneous administration of enalapril significantly reduced the thickness of submesothelial connective tissue, produced fewer adhesions, and was associated with lower concentration of tumor growth factor-β (TGF-β). Studies on cultured HPMC additionally showed that TGF-β1 induced by high glucose is controlled by angiotensin-converting enzyme inhibition and angiotensin II receptor blocker [68].

In rats dexamethasone has a diminishing effect on the fibroproliferative phase of non-inflammatory TGF-β-induced peritoneal fibrosis [69]. Rapamycin, an antirejection agent that has potential antifibrotic and anti-angiogenic activity, used intraperitoneally in a rodent model of TGF-β1-induced peritoneal fibrosis and angiogenesis, did not have significant benefit on the morphological changes in the peritoneum [70].

The high glucose concentrations of the dialysis solutions may saturate physiological glucose metabolism pathways and stimulate the polyol pathway, which probably contributes in the development of fibrosis and angiogenesis during peritoneal dialysis. In this pathway of intracellular glucose metabolism, glucose is reduced to sorbitol by aldose reductase, coupled with oxidation of NADPH to $NADP^+$. Sorbitol is then oxidized to fructose by sorbitol dehydrogenase, coupled with reduction of NAD^+ to NADH. Possible mechanisms of polyol pathway-linked functional abnormalities include osmotic stress due to intracellular accumulation of sorbitol, an increased NADH/NAD^+ ratio leading to pseudohypoxia, and enhancement of the formation of AGEs by fructose. Inhibition of the polyol pathway in rats by administration of zopolrestat, a newly developed inhibitor of aldose reductase activity, resulted in less fibrosis and fewer peritoneal vessels than in rats dialyzed with 3.86% glucose-containing fluid without zopolrestat [71].

An antioxidant, sodium sulfite, is an additive commonly used for food preservation. It was able to suppress AGEs formation in rats with normal renal function, eliminating oxidative stress caused by methylglyoxal. It is presumed that sodium sulfite reacts with methylglyoxal to form chemically inactive substances. Sodium sulfite was administered intraperitoneally to rats once a day for 5 consecutive days together with methylglyoxal. Other group of rats was given methylglyoxal alone. Prominent hypervascularity and intense immunostaining of anti-AGE antibodies were noted in methylglyoxal-treated rats, whereas the macroscopic alterations were suppressed in the rats that had been treated with sodium sulfite [72].

The potential use of AGEs inhibitors and breakers as salvage therapy for peritoneal membrane failure was also considered in studies with pimagedine, which has been shown to inhibit the formation of AGEs and to slow the progression of nephropathy in animal models [73].

Decreasing infection rate and its harmful consequences

Severe or repeated episodes of peritonitis are particularly damaging to the peritoneal membrane. The short-term, single episodes had no significant effect on membrane permeability or ultrafiltration, while recurrences or clusters of infection caused an increase in membrane permeability and reductions in ultrafiltration. Thus, prevention of infectious complications of peritoneal dialysis, especially peritonitis, is a great challenge for every dialysis unit. Proper patient' education and regular use of mupirocin at the exit site exert an important role in diminishing peritonitis rate. Use of prophylactic antibiotics at the time of catheter insertion has also been shown to reduce the incidence of early peritonitis [74]. Accepted rate of peritonitis is 1 episode per 18 patient-months [75].

In the rat model of peritoneal dialysis it was shown that hyaluronan modifies inflammatory response and peritoneal permeability during peritonitis [48,53,76]. There are no studies confirming this beneficial effect in humans.

It was shown that also acute systemic inflammation influences the peritoneal membrane function, increasing small solute transport rate. Possible mechanisms linking inflammation and peritoneal transport include enhancement of vascular superoxide formation leading to modification of endothelial junctional elements (advanced oxidation protein products formation), IL-6 increased generation influencing D/P of creatinine and a direct effect of C-reative protein on vascular permeability [77]. This finding may contribute to explanation of reasons of damage of the peritoneal membrane over dialysis duration in patients without peritonitis, but showing episodes of systemic inflammation. Thus, to protect the peritoneal membrane one also has to pay attention for avoiding systemic inflammation.

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Realization of International Healthy Hearing Program in Poland – hearing evaluation in participants of Special Olympics

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Abstract

Purpose: International Healthy Hearing Program developed by International Special Olympics in Washington DC performs hearing screening during athletics competitions of athletes with mental retardation. The aim of this study was to introduce hearing screening performed according to Special Olympics Incorporated (SOI) Healthy Athletes Program.

Material and methods: The study was performed in Polish participans of Special Olympics during Summer National Special Olympics Game in Olsztyn in 2005 and Winter National Special Olympics Game in Białystok in 2004. HH evaluation was divided into 4 screening sequences: otoscopy, otoacoustic emission (DPOAE), tympanometry and pure-tone audiometry. During athletics competitions 208 Polish participants were examined.

Results: Of the total 208 athletes screened: 156 passed OAE (75%), 42 passed pure tone screening at 25 dB HL (20.2%), and 5 more passed the pure tone threshold test (2.4%). It means total of 203 passing hearing testing (97.6%). Hearing impairments were detected in 4.8% athletes and 2.4% of athletes needed hearing aids.

Conclusions: HH Program provided a more precise analysis of hearing in the group of athletes with mental retardation and a recognition of subjects who need audiological care.

Key words: mental retardation, Special Olympics athletes, Healthy Hearing Program.

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Introduction

The mission of the Special Olympics Incorporated (SOI) Healthy Athletes Program, which is connected with the activity of International Special Olympics Committee, is to improve each athlete's ability to train and compete in Special Olympics Games. Officially launched in 1996, the Special Olympics Healthy Athletes Program regularly provides health services to Special Olympics athletes in two health specialties: dentistry and otometry. At the 1999 Special Olympics Healthy Athletes Program introduced health screening opportunities in hearing, nutrition, dermatology, physical therapy and orthopedics. The key objectives of the Healthy Athletes Program, under the auspices of Eunice Kennedy Shriver, Founder and Honorary Chairman and Timothy P. Shriver, Ph.D., President and CEO are: 1) to improve access and health care for athletes at event-based health clinics, 2) to train and educate health care professionals and students about the special needs of, how to communicate with, and care for, people with mental retardation, 3) to collect and analyze data and communicate about the health conditions and needs of people with mental retardation, 4) to raise public and professional awareness of the health care problems that Special Olympics athletes face.

The Healthy Hearing (HH) Program is a primary component of the Special Olympics Healthy Athletes Program and is designed for two purposes:

1. To study the prevalence of hearing loss in athletes competing in sport events,
2. To screen the hearing of athletes who participate in particular events, and notify them and their coaches and families if follow-up care is needed.

The most commonly cited definition of mental retardation comes from the American Association of Mental Retardation (AAMR). The AAMR has defined MR as the onset of significant limitations in both general intellectual and adaptive functioning during the developmental period (18 years and under). Intellectual limitation refers to an IQ which falls two standard deviations below the population mean (IQ<70) [1,2]. MR is

Table 1. **Results of test according to HH Program in screened group**

Examinations	Results					
OTOSCOPY	**208 athletes** → 114 (54,8%) external canal partially blocked by cerumen → 62 (29,8%) features of chronic eustachitis → 29 (14%) recurrent infections of upper respiratory tract					
DPOAE	+156 (75%)		-52 (25%)			
TYMPANOMETRY SCREEN			+31 (14.9%)		-21 (10.1%)	
PURE TONE SCREEN			+31 (14.9%)	-0	+11 (5.3%)	-10 (4.8%)
PURE TONE THRESHOLD TEST						+5 (2.4%) -5 (2.4%)
HEARING SCREEN	+203 (97.6%)					-5 (2.4%)

\+ Pass; - No pass
* 1) Otoscope Riester CE, Periscope; 2) Euro-Scan No 1824009 Widex, Maiko; 3) Maiko – Mi 24 Oticon; 4) Audio Screen Madsen Electronics

also defined by the American Psychiatric Association (APA) – Diagnostic and Statistical Manual of Mental Disorders (DSM--IV). The DSM-IV defined the degrees of MR: mild (IQ =50-55 to 70), moderate (IQ =34-40 to 50-55), severe (IQ =20-25 to 35-40) and profound (IQ <20-25) [3].

The aim of this study was to introduce hearing screening performed during Summer National Special Olympics Game in Olsztyn in 2005 and Winter National Special Olympics Game in Białystok in 2004 for Polish participants, according to Special Olympics Incorporated (SOI) Healthy Athletes Program.

Material and methods

The first two events in Poland which offered screening of hearing according to directives of Healthy Hearing Program (Herer & Montgomery, 2001) were performed with a group of 208 Polish athletes with mental retardation ages 18-44 during Summer National Special Olympics Game in Olsztyn in 2005 (130 participants) and Winter National Special Olympics Game in Białystok in 2004 (78 participants).

The HH Program contained four screening sequences: otoscopy, otoacoustic emission (DPOAE), tympanometry and pure-tone audiometry. Athletes were also conducted through two registration/check-out desks. First, before starting hearing screening, the purposes and techniques of screening were explained to each participant and coach or accompanying person. Using the report form, diseases of the upper respiratory tract and the most relevant information in the athletes' medical histories were gathered from coaches or accompanying persons. Next the laryngological examination of the pharynx, larynx, nose for elimination of the upper respiratory tract infections and developmental or anatomical anomalies were performed.

HH evaluation was divided into 4 screening sequences: otoscopy, otoacoustic emission (DPOAE), tympanometry and pure-tone audiometry. The pure tone threshold test was performed only in these cases in which every examination was failed. All examinations occured in the special testing area called "silence zone" which is, as a rule, a separate room near the gym or classroom. The following types of equipment were used for screening athletes:

1* –for otoscopy: PLEASE INSERT EQUIPMENT NAME AND NUMBER FOR EACH
2 – for otoacoustic emission DPOAE:
3 – for tympanometry:
4 – for pure-tone audiometry:

The first screening station (STATION 1 – otoscopy) examined ear canals and tympanic membranes tympanic for the presence of cerumen and signs of infections. Using the report form, volunteers circled if the external ear was clear or partially blocked. If something was unusual or ear canal is blocked the Clinical Director was alerted and decided about the athlete participating in the Special Olympics game. The second one (STATION 2 – otoacoustic emission DPOAE) screened hearing at 2000, 3000, 4000 and 5000 Hz using distortion product otoacoustic emission screener for objective estimation of inner ear (cochlear part of hearing organ). If an athlete passed the second station of the hearing screen, he proceeded to the check-out desk, turned in the screening report form and received a copy of the results. If an athlete did not pass the DPOAE he went to the third station (STATION 3 – tympamometry) and then the fourth station (STATION 4 – pure-tone audiometry). Tympanometry instrumentation objectively screens for middle ear conditions that may result in conductive hearing loss and thus may explain why the athlete did not pass the initial DPOAE hearing screen. The pure tone screen evaluated hearing acuity at 2000, 4000 Hz at 25 dB Hearing Level (HL) according to directives of Special Olympics Healthy Hearing Program after training tone of 50 dB HL. If a person indicated hearing each test tone, usually giving the behavioral response of raising a hand, then hearing thresholds were considered normal and that person should have no difficulty listening to the speech of others. The pure tone screen served to confirm the DPOAE screening outcome. The athlete completing the fourth screen then proceeded to the check-out desk, received a copy of the report form, which included follow-up medical or audiological recommendations as needed.

Results

In the examined Polish Special Olympics athletes, 177 of 208 participants (85.1%) had mild degree of mental retardation according to APA and 31 of 208 (14.9%) had moderate degree of MR.

The audiologic screening, performed according to directives of Healthy Hearing Program, in group of 208 athletes with MR disclosed (*Tab. 1*):

– 203 (97.6%) passed hearing screen,

– 114 (54.8%) athletes had ear canals partially blocked by cerumen,

– 62 of 208 (29.8%) had features of chronic eustachitis and 29 (14%) of them had recurrent infections of upper respiratory tract,

– 52 of 208 (25%) failed otoacoustic emission (DPOAE) screen and 156 athletes (75%) pass hearing screen,

– 21 of 52 (40.4%) of the athletes who failed DPOAE also tympanometry (21/208=10.1% overall),

– Among those 21 athletes – 11 (11/208=5.3%) passed pure tone screen at 25 dB HL,

– 10 of 52 (19.2%) had not passed pure tone screen at 25 dB HL (10/208=4.8%) and hearing loss concerned both ears. The 10 went on to receive pure tone threshold where 5 showed threshold losses (5/208=2.4%), and 5 passed their threshold hearing test. These athletes showed hearing loss/hearing aid needs,

– The 5 athletes who showed "hearing loss/hearing aid needs" were among those also failing tympanometry,

– Of the total 208 athletes screened: 156 passed OAE (75%), 42 passed pure tone screening at 25 dB HL (20.2%), and 5 more passed the pure tone threshold test (2.4%) for a total of 203 passing hearing testing – 97.6%.

Examination were performed by six trained and supervised volunteers (students of the Medical School).

Discussion

In an examined population of Special Olympics athletes diseases of middle ear and hearing disturbances occurred more often than in the general population. Athletes with mental retardation are particularly susceptible to otitis media or middle ear infections because they have developing immune systems which often show difficulty fighting infections, immature Eustachian tubes that prevent optimal fluid drainage, and may have enlarged adenoids that interfere with the Eustachian tube opening. Otitis media if it remains untreated can cause permanent hearing loss. Additionally, recurrent otitis media can have a negative impact on speech and language development, cognitive achievement and social and emotional development.

The prevalence of otitis media among people with mental retardation has not been adequately explored. There are some reasons to believe that persons with Down Syndrome are at increased risk of middle ear infections due to midfacial malformations and increased susceptibility to infections [5]. Dahle and McCollister [6] compared the prevalence of ear problems in persons with Down Syndrome to those with other forms of MR. They found that hearing impairment and infections were more prevalent among persons with Down Syndrome. Although not focused specifically on otitis media, the study of 293 residents of an English institution found that 40% of individuals with Down Syndrome and 29% of individuals with other MR had ear, nose and throat conditions [7]. Whiteman's research in group of person with MR confirmed that they are at risk for otitis media and middle ear infections [8].

Systematic audiologic examination in a population of athletes with MR is the first step in the process of identifying an athlete's hearing loss and preventing its negative effects from occurring in sporting and social events. Data gathered during European and World SO Games in period of 1999-2003 by Montgomery and Herer [9] disclosed that whereas less than 2% of adults ages 18-44 have hearing loss in USA, only 74% of the participants examined at Special Olympics Games showed positive results of hearing screen. Our study performed in Polish athletes during XI International Summer Special Olympics Games in Dublin in 2003 were similar (2.5% participants have hearing loss). Thus Healthy Hearing Program accomplished the mission of the Special Olympics Healthy Athletes Program of improving, through better health, each athlete's ability to train and compete in Special Olympics.

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Tumor front grading in prediction of survival and lymph node metastases in patients with laryngeal carcinoma

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Abstract

Purpose: Despite innumerous both therapeutic and histopathologic studies that have been performed no morphologic markers are currently available in order to predict the outcome in patients with laryngeal cancer. According to the recent reports nowadays tumor front grading (TFG) is one of the most reliable methods of estimation of the progress of the changes in the peripheral part of tumor and it seems to be one of the technics, which is able to assess the dynamics of the tumor growth quite precisely. In this sudy it was presented direct relation between morphological features of tumor front and survival.

Material and methods: The authors have analysed 120 cases of patients who were operated on the laryngeal squamous cell carcinoma in ENT Department Medical University of Łódź between 1995-2000. Features of the morphologic tumor front grading was performed on H&E-stained sections in the peripheral parts of a tumor. Dependence on tumor grade G, tumor size T, lymph node metastases and survival were analysed.

Results: Our study showed that feature such as TFG is very useful in prediction of survival in patients with laryngeal squamous cell carcinoma in comparison to the histological differentiation degree. The statistical analysis showed no significant correlation between TFG score and tumor size T, nodal status N and G feature.

Conclusions: The presented study emphasizes that TFG might influence decisions regarding therapeutic management and could eventually lead to more appropriate and individualized therapy. It is necessary to extend the traditional histopathological diagnosis by TFG, which assesses the dynamics of the malignant process and it seems to be a good prognostic method in prediction of survival of patients with squamous cell carcinoma.

Key words: laryngeal carcinoma, tumor front grading, lymph node metastases, disease-free survival time, corrected actuarial survival.

Introduction

The cure rates of squamous cell carcinoma of the larynx have scarcely improved over past few decades. Despite innumerous both therapeutic and histopathologic studies that have been performed no morphologic markers are currently available in order to predict the outcome in patients with laryngeal cancer. The essential meaning of the estimation of the advance of the cancerous process and the choice of appropriate surgical treatment and prediction of survival and recurrences has been performed due to the histopatological examination [1-8]. This prognosis method considers only histological differentiation degree G and cancerous advance established on the basis of TNM classification. Traditional Broder's grading system, recently the most widely used method of staging disease, is not satisfactory in predicting survival and prognosis [1,3]. Scientists have been looking for a new diagnostic method, which could help to take a decision as to the basic and supplementary treatment, to predict metastases and survival, or the modification of already existing methods. According to the recent reports, nowadays tumor front grading (TFG) is one of the most reliable methods of estimation of the progress of the changes in the peripheral part of the tumor and it seems to be one of the technics, which is able to assess the dynamics of the tumor growth quite precisely [4,8].

The purpose of this study was to analyse the tumor biologic factors in patients who were operated on the laryngeal squamous cell carcinoma and to determine the relationship between morphologic grading at the tumor front and the tumor grade

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***Table 1*. Features used for tumor front grading and numeric score**

Features	Score points			
	1	2	3	4
Cytoplasmic differentiation	high (>50% keratinized)	moderate (20-50% keratinized)	poor (5-20% keratinized)	none (<5% keratinized)
Nuclear differentiation (polymorphism)	high (>75% mature cells)	moderate (50-75% mature cells)	poor (25-50% mature cells)	none (<25% mature cells)
Number of mitoses	single (0-1)	moderate number (2-3)	large number (4-5)	very numerous (>5)
Mode of invasion	well-defined bordeline	cords; less marked bordeline	groups of cells; no distinct bordeline	diffuse growth
Stage of invasion (depth)	possible invasion	microinvasion (few cords)	nodular into submucosa	invasion deper than submucosa
Plasmalymphocytic invasion	marked (continous rim)	moderate (many large patches)	slight (few small patches)	none

G estimated in the routine histology, tumor size T, lymph node N+ and N- and to the recurrence-free survival time and the corrected actuarial survival.

Material and methods

120 patients with histologically recognized squamous cell carcinoma were treated surgically from 1995 to 2000 in ENT Department Medical University of Łódź. The examination of these patients' specimens included routine histology and morphologic tumor front grading. Only the cases with follow-up at least 24 months were included.

In order to routine histology parrafin sections were stained with hetoxylin and eosin (H&E) and examined by the pathologist. Tumor were graded according to morphologic differentiation as grade G1 – well differentiated, grade G2 – moderately well differentiated, grade G3 – poorly differentiated and grade G4 – undifferentiated.

Morphologic tumor front grading was performed on H&E--stained sections in the peripheral parts of a tumor. We analysed the following features in the most invasive zones of the tumor: the cytoplasmic differentiation, the nuclear polymorphism, the number of mitoses per high power field, the mode of infiltration, the depth of invasion and the plasmalymphocytic infiltration of tumor front. These factors were assessed in at least five different regions of the peripheral part of the tumor (x400). Each factor was graded according to a scale ranging from 1 to 4. The total morphologic tumor front grading score was computed as the sum of the six parameters, with maximum score of 24 points. To compare the survival with the TFG total score the patients were divided into 4 groups: 6-9 points, 10-13 points, 14-17 points and 18-21 points of TFG (*Tab. 1*).

The statistical analysis was performed to determine whether the TFG score is correlated with survival. All statistical analyses were carried out with the aid of the χ^2 test, Spearman's test, Mann-Whiney's test or t-Student's test. The corrected survival curves were calculated according to the actuarial method of Kaplan and Meier. In all cases, only p values of less than 0.05 were taken to indicate statistical significance.

Results

Specimens taken from 120 patients with laryngeal carcinoma (104 men, 16 women, with an average age of 57.4 years and age range from 39 to 74 years) were examined. All patients underwent surgery – 75 (62.5%) patients out of 120 underwent selective neck dissection (SND), 35 (29.1%) radical neck dissection (RND), mainly ipsilateral, 5 (4.2%) contralateral SND and ipsilateral RND next and 5 (4.2%) ipsilateral RND following ipsilateral selective neck dissection.

The patients were classified according to the basis of TNM Classification of WHO (1990) International Classification of Diseases for Oncology. In this study 70 (58.4%) patients presented supraglottic and glottic tumors, 35 (29.1%) translaryngeal tumors, 10 (8.3%) supraglottic tumors and 5 (4.2%) glottic and subglottic tumors, with stages from II to IV represented. In the examined group there were 10 (8.3%) patients with T2 tumor, 25 (20.8%) with T3 tumor and 85 (70.9%) with T4 one. In the case of 45 (37.5%) patients out of 120 subclinical cervical adenopathy was not detected through histological examination of the removed tissues, and the remaining 75 (62.5%) patients with lymph node metastases stadium N1 in 25 (20.8%), N2 in 50 (41.7%) patients was evaluated. There were no patients with N3 nodal status.

The invasive squamous cell carcinoma was confirmed histologically in all cases. In the examined group there were 20 (16.7%) patients with G1 carcinoma, 55 (45.8%) in G2 and 45 (37%) in G3 carcinoma. The undifferentiated carcinoma was not observed.

In the present study the analysis of tumor front grading revealed that the most numerous of tumors were carcinomas with the average TFG score (10-13 points) – 44.5%.

The actuarial corrected survival for the total group of patients was 35.4% after 5 years of follow-up. Analysis of the influence of the TFG score on the actuarial corrected survival revealed that the tumor front grading had a significant impact on the prognosis of survival (*Fig. 1*). Analysis of each feature used for morphologic grading revealed that depth and mode of invasion had significant influence on corrected survival. Histopathologically proven diffuse invasion of the thyroid cartilage

Figure 1. **Actuarial corrected survival for total tumor front grading (TFG) score**

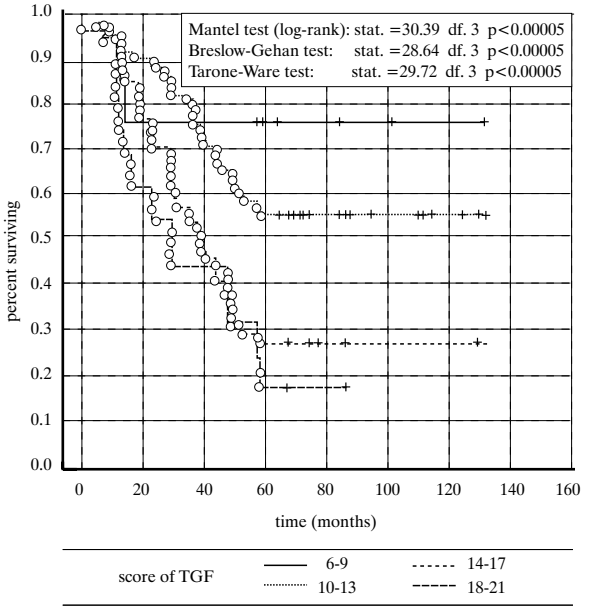


Figure 2. **Actuarial corrected survival related to depth of invasion**

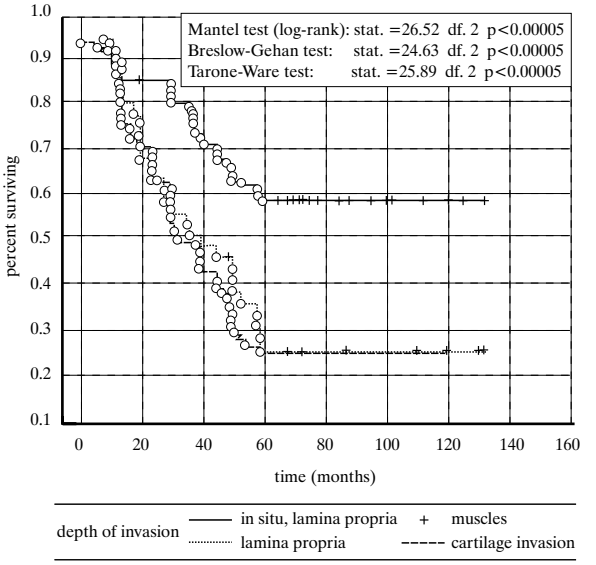


Figure 3. **Actuarial corrected survival related to mode of invasion**

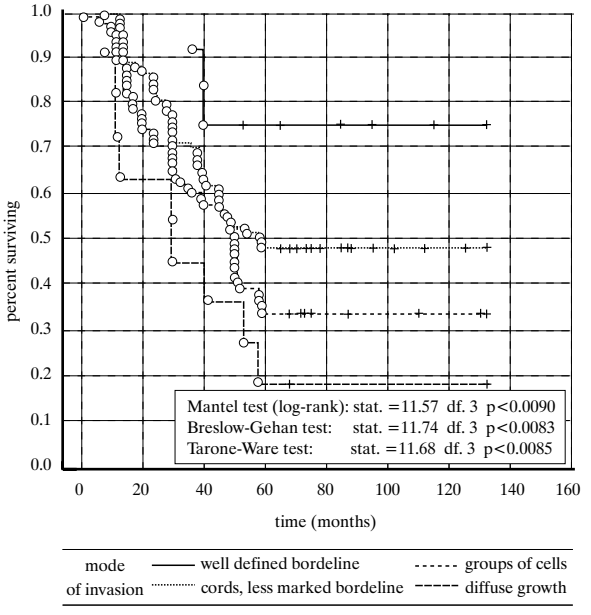
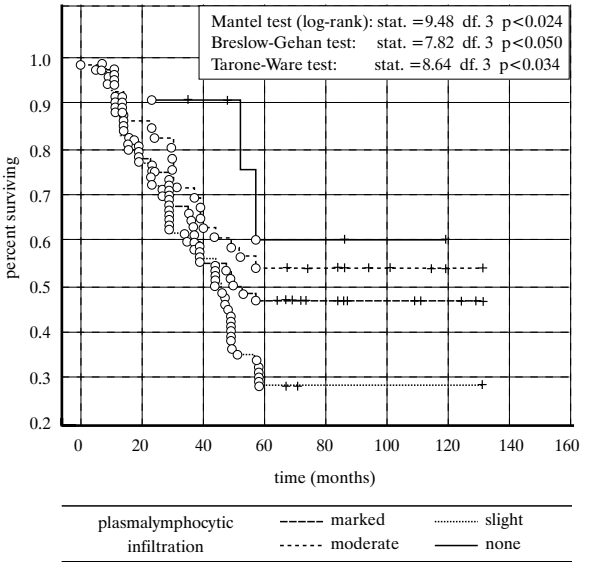


Figure 4. **Actuarial corrected survival for plasmalymphocytic infiltration**



resulted in a significant decrease in the corrected survival rate (*Fig. 2*, *3*). The presence of plasmalymphocytic infiltration of the tumor space had a significant impact on the prognosis of survival as well (*Fig. 4*). Number of mitoses per high-power field did not demonstrate prognostic value on corrected survival (*Fig. 5*).

The patients were also divided into two groups. First of which was represented by patients with the recurrence-free survival time under 5 years – 90 (75%), and the second group with survival of 5 years and over 5 years – 30 (25%).

Our study showed that feature such as TFG is very useful in prediction of survival and recurrence in patients with laryngeal squamous cell carcinoma in comparison to the histological differentiation degree. The mean number of points of TFG feature was 14.3±3 (range 6 to 21). In 90 patients with survival under 5 years, TFG feature was meanly 12.1±2.5 and in remaining ones with survival of 5 years and over 5 years TFG was 14.8±3.2. The difference between these both group was statistically significant ($p<0.05$) (*Tab. 2*).

The statistical analysis showed no significant correlation between summary number of points for TFG and tumor size T and G feature. Only for G3 tumors TFG scores demonstrated higer values. The mean number of points of TFG for T4 was

Figure 5. **Actuarial corrected survival for number of mitoses per high-power field**

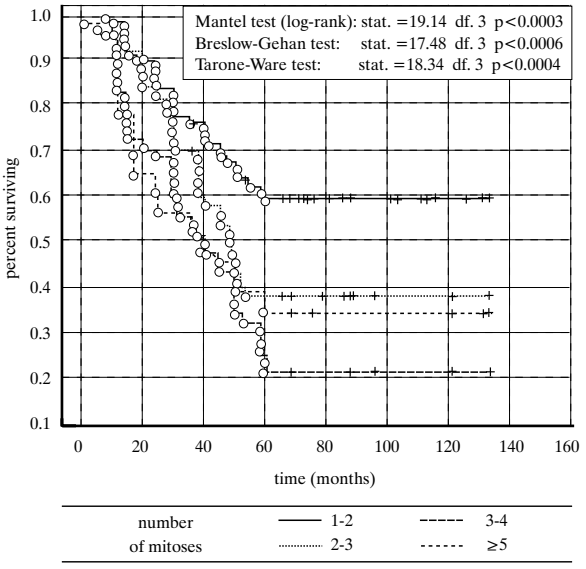


Table 2. **Dependence on G, T, N, disease-free survival time upon TFG**

Feature	Number of cases (%)	TFG (+/- SD)
G1	20 (16.7)	12.3 (+/-2.2)
G2	55 (45.8)	13.2 (+/-3.8)
G3	45 (37.5)	17.4 (+/-2)
T2	10 (8.3)	14.1 (+/-1.2)
T3	25 (20.8)	14.4 (+/-3.1)
T4	95 (79.2)	15.5 (+/-4.2)
N+	25 (20.8)	15.1 (+/-3.5)
N-	50 (41.7)	14.8 (+/-4)
<5-years survival time	90 (75)	12.1 (+/-2.5)
≥5-years survival time	30 (25)	14.8 (+/-3.2)

15.5±4.2 , for T3 tumors was 14.4±3.1 and for T2 was 14.1±1.2, for G1 was 12.3±2.2, for G2 13.2±3.8 and for G3 tumors was 17.4±2.

No significant differences were found between TFG and neck lymph node status N. Mean number of points for group with subclinical cervical adenopathy (N+) and without lymph node metastases one (N-) was much the same and amounted for N+ 15.1±3.5 and for N- 14.8±4.

Discussion

From the factors relevant to prognosis of laryngeal carcinomas such as tumor site, tumor stage and nodal status, the last one is one of the most important traditional prognostic factors in laryngeal squamous cell carcinoma which influences the recurrence rate after the previous treatment [7-11]. However, on the basis of the several studies performed recently, biologic factors or host-related factors probably play the most important role in determining the eventual disease outcome [4-6,8,12].

Tumor front grading seems to be a good prognostic method of survival in patients with laryngeal carcinoma in comparison with traditional diagnostic methods [4,8]. Routinely used histologic differentation degree has limited application because of only partly correlation with cancerous process [5]. In accordance with the results of TFG in squamous cell carcinoma of other sites such as oral cavity, tongue and breast the new malignancy grading system is used for both prediction recurrences and patient survival [2,13]. TFG is the technique, which assesses the dynamics of the tumor growth and provides multifactorial morphologic information about the carcinoma tissue. Cell differentiation, nuclear polymorphism, and number of mitoses are directly related to the biology of the tumor cells. Apart from elements of traditional histologic grading TFG also includes a type and depth of infiltration of the cancer, two elements, which indicate the possibility of microfocal malignant invasion – the tumor aggressiveness. The lymphocytic infiltration might be related to the immunologic defense of the host.

Our results of the high correlation between TFG grading system and survival in patients with laryngeal cancer are in accordance with the other authors' results of tumor front grading in squamous cell carcinoma of other sites, including the oral cavity and tongue [2,4,8,13]. Therefore we suggest that prognosis should be determined by the biology of the cells at the most invasive zone of the tumor and it seems that histopathologic routine examinations should be extended by analysis of TFG as a suplement of traditional histologic estimation.

In histopathologic researches features as cell differentiation, nuclear polymorphism, number of mitoses, type and depth of infiltration appear as elements of many classifications and ranges of primary tumor [12-16]. Many authors have undertaken multifactorial morphologic valuation of the tumor in squamous cell carcinoma of other sites introduced similar conclusion [12--16]. Anneroth, Batsakis and Luna [14] separated the patients group with better prognosis using their own classification. Trial of prognosis in patients with laryngeal carcinoma undertake Jacobsson [12]. Author indicated total score in his classification is the prognostic factor and the most important features are nuclear polymorphism and mode of invasion. Zatterstrom [16] used Jacobson's scale has not proved relationship between histologic grading and clinical features. Crissman and Zarbo [15] proposed own modified morphologic scale and total score has the prognostic value. Other authors have not accepted Jacobson's and Crissman's classifications [13,17]. Lund [13,17] precisely defined every degree for histologic feature and proved that this scale correlated with survival and clinical disease course. Many researchers indicate the difference between central and periferal part of the tumor [4,8,15]. Welkoborsky [8] disclosed high correlation total score of tumor front grading with disease-free survival and local carcinoma recurrens. Similar conclusions introduced Gabriel [4] which estimated the necessity to add TFG to traditional histopatologic diagnosis.

The presented study emphasizes that TFG might influence decisions regarding therapeutic management and could eventually lead to more appropriate and individualized therapy. It is necessary to extend the traditional histopathological diagnosis

by TFG, which assesses the dynamics of the malignant process and it seems to be a good prognostic method in prediction of survival of patients with squamous cell carcinoma.

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Activity of coagulation and fibrinolytic system components in the vein thrombus

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Abstract

Purpose: Behaviour of the vein thrombus is determined by the activity ratio of coagulation factors to factors of fibrinolytic system. The aim of the study is to evaluate activity of some coagulation and fibrinolytic factors in the vein thrombus.

Material and methods: The activity of platelets aggregating factors, tissue factor, thrombin, antithrombins, antiheparin factors, plasminogen activators, plasmin (plasminogen) and antiplasmins of the vein thrombus homogenate was determined using coagulative, fibrinolytic and caseinolytic tests. Retracted blood clot was a compared material.

Results: Tissue factor activity in the vein thrombus was above twofold higher and antiheparin activity was nearly twice higher in comparison to the blood clot. The vein thrombus contains also active thrombin. Plasminogen activators activity in the vein thrombus was twofold higher and activity of plasmin (plasminogen) was threefold higher than in the blood clot. High activity of the tissue factor, substances neutralizing heparin and presence of thrombin intensify the thrombus enlargement. However, the thrombotic tendency may be balanced by a high activity of plasminogen activators and high activity of plasmin (plasminogen).

Conclusions:

1) Vein thrombus is characterized by high activity of tissue factor, presence of active thrombin and high antiheparin activity.

2) High coagulative potential of vein thrombus is balanced to a certain grade by high fibrinolytic potential: high activity of plasminogen activators and high activity of plasmin (plasminogen), as well as absence of antiplasmins activity.

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Introduction

The vein wall during thrombophlebitis shows higher activity of the tissue factor and lower activity of the plasminogen activators than the wall of unchanged vein [1]. Interaction of coagulation factors of the inflammatory changed vein wall with plasma coagulation factors, platelets and leucocytes results in formation of parietal thrombus [2,3]. Further thrombus condition is determined by the relation of coagulation factors activity to activity of fibrinolytic system factors. Activity of the vein thrombus haemostatic system components is still unknown.

The aim of this report is to evaluate the activity of platelets aggregating factors, tissue factor, thrombin, antithrombins, antiheparin factors, plasminogen activators, plasmin (plasminogen) and antiplasmins of the vein thrombus homogenate. Coagulation, fibrinolytic and caseinolytic methods were used to determine the factors mentioned above. Plasma coagulation and fibrinolytic factors, including strategic substrates for the haemostatic system such as blood platelets, fibrinogen and fibrin were the substrates for the vein thrombus haemostatic components. The employed methods allow evaluating the real effect of thrombus haemostatic factors on the coagulation – anticoagulation balance in circulating blood so important in examinations of pathobiochemistry and in diagnosis and treatment of venous thromboembolic disease.

Material and methods

Parietal thrombi from varicose saphenous veins in the course of thrombophlebitis were taken during surgery from 8 patients (5 men and 3 women) aged 33-48 years. Thrombosis of the proximal part of the saphenous vein was an indication for operation. Unfractionated and low molecular weight heparins, as well as

antiplatelets drugs were not administered to patients 7 days before and during the operation. The risk factors of venous thromboembolic disease, such as laboratory indicators of thrombophilia and previous deep veins thrombosis were not found in these patients. The signs of circulatory, liver and kidney dysfunction were also absent. The control material consisted of 8 retracted blood clots obtained *in vitro* from blood collected before the operation from the same patients. Thrombi and clots were stored at -70°C. They were thawed directly before the measurements in water with ice and homogenized in 0.15 mol/l NaCl (1:9 w/v) with the use of flow homogeniser. The supernatant obtained by centrifugation (2700 x g, 30 min, 2°C) was used for the examinations.

Reagents: Palitsch borate buffer [4]; fibrinogen, heparin, plasmin, streptokinase and thrombin, Sigma, USA; hirudin, Hoechst AG, Germany; casein, BDH , England; Folin and Ciocalteau reagent, Merk, Germany; substrate platelets rich plasma (250000 platelets/mm^3) and substrate platelets poor plasma obtained from blood taken into 0.1 mol/l sodium citrate (9:1 v/v) and centrifuged at 2°C (500 x g during 5 minutes and 2700 x g during 30 minutes respectively).

1. Activity of the platelet aggregating factors was evaluated by means of the turbidimetric method [5].

0.1 ml of the homogenate and 0.1 ml 0.15 mol/l NaCl or 0.1 ml 50 μg/ml hirudin was added to 0.9 ml of platelets rich plasma and the time of platelet aggregates appearance manifested by decrease of turbidance was measured. The time elapse was a measure of the aggregating factor activity.

2. Tissue factor activity was evaluated by means of the coagulation method [6].

0.1 ml of the homogenate (in controls 0.15 mol/l NaCl) and 0.1 ml 0.5 mol/l $CaCl_2$ were added to 0.2 ml of platelets poor plasma and the coagulation time at 37°C was measured. Shortening of the coagulation time as compared to the controls was accepted as a measure of the tissue factor activity.

3. Thrombin activity was evaluated using fibrinogen [7].

0.1 ml 0.15 mol/l NaCl or 0.1 ml 1.5 μg/ml of hirudin and 0.1 ml of the homogenate, was added to 0.2 ml 0.3% fibrinogen and the coagulation time was measured at 37°C. The fibrinogen coagulation time was a measure of the thrombin activity.

4. Antithrombin activity was evaluated by means of the coagulation method [7].

0.1 ml of the homogenate (in controls 0.1 mol/l NaCl) and 0.1 ml of thrombin (8 u/ml) were added to 0.2 ml of the platelet poor plasma and the coagulation time at 37°C was measured. Prolongation of the coagulation time as compared to the controls was accepted as a measure of the antithrombin activity.

5. Antiheparin activity was determined with use of heparin-thrombin test [8].

0.1 ml of heparin (2.5 u/ml), 0.3 ml of the platelets poor plasma were added to 0.1 ml of the homogenate (in controls 0.15 mol/l NaCl) and preincubated at 37°C for 5 minutes. The coagulation time was measured after adding 0.1 ml of thrombin (40 u/ml). Shortening of the coagulation time as compared to controls was accepted as a measure of antiheparin activity.

6. Activity of the plasminogen activators was evaluated by means of the euglobulin method [6].

0.5 ml of the platelets poor plasma and 0.25 ml of the homogenate (in controls 0.25 ml of 0.15 mol/l NaCl) were added to 10 ml of cooled distilled water acidized to pH 5.3 by acetic acid. The samples were kept at 2°C for one hour. The sediment obtained by centrifugation (2700 x g, 30 minutes, 2°C) was dissolved in 0.5 ml borate buffer. 0.5 ml of 0.025 mol/l $CaCl_2$ containing thrombin (1 u/ml) was added to the samples and the fibrinolysis time of the formed clot was measured in the temperature of 37°C. Shortening of the fibrinolysis time as compared to the controls was accepted as a measure of the plasminogen activators activity.

7. The plasmin activity (after plasminogen activation by streptokinase) was determined by means of the caseinolytic method [9].

0.25 ml of 0.16 mol/l HCl was added to 0.25 ml of homogenate and incubated at 20°C for 15 minutes. After that 0.25 ml of 0.16 mol/l NaOH, 0.2 ml of borate buffer and 0.05 ml of streptokinase (10000 u/ml) were added and preincubated at 37°C for 15 minutes. Then 1 ml of 2% casein was added and the samples were incubated at 37°C for 60 minutes. The reaction was stopped by adding 1 ml of 15% trichloroacetic acid. The samples precipitated in 0 time were accepted as controls. Tyrosine was determined in the clear supernatant obtained by centrifugation by means of Folin and Ciocialteau reagent. The activated plasminogen content was expressed by the increase of quantity of released tyrosine.

8. Activity of antiplasmins was determined by means of the caseinolytic method [10].

0.25 ml of the homogenate (in controls 0.15 mol/l NaCl) was added to 0.25 ml of 0.1% plasmin and preincubated at 37°C for 15 minutes. After that 0.5 ml of 2% casein was added and incubated at 37°C for 60 minutes. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid. Further procedures were as in point 7. Activity of antiplasmins was expressed by the decrease of released tyrosine as compared to controls.

9. The protein content was determined by means of Lowry method [11].

Mean values ±SD of the obtained results are presented. Results obtained for the vein thrombus and blood clot were statistically analyzed by t-Student test, accepting $p<0.05$ as statistically significant.

Results

Blood platelet aggregating factors are present in about 50% of the vein thrombi. They are not found in the blood clot obtained *in vitro* (*Tab. 1*). Activity of blood platelet aggregating factors is cancelled by addition of hirudin. Tissue factor activity of the vein thrombus is above twofold higher than in the blood clot. Active thrombin is found in about 50% of the vein thrombi. Its activity is cancelled by hirudin. Antithrombin activity is not observed in vein thrombus. Antiheparin activity in the vein thrombus is nearly twofold higher than in the blood clot obtained *in vitro*. The activity of plasminogen activators in the vein thrombus is twofold higher than in the blood clot. Plasmin (plasminogen) activity in the vein thrombus is threefold higher than in the blood clot. Antiplasmins activity in the vein thrombus is absent and it is small in the blood clot. The vein thrombus contains less protein than the blood clot.

Table 1. **Activity of coagulation and fibrinolytic systems components in vein thrombus and blood clot**

Determined factor	Control	Vein thrombus	Blood clot
Activity of platelet aggregating factors, s		127.2±14.6	>10 min
Tissue factor activity, s	120.5±12.3	36.5±4.0*	84.6±9.4
Thrombin activity, s		40.1±4.2	>10 min
Antithrombin activity, s	15.6±1.2	14.5±2.0	15.3±1.4
Antiheparin activity, s	32.0±2.6	18.6±1.2*	23.7±2.4
Plasminogen activators activity, min	225.0±2.04	52.8±6.7*	96.5±12.0
Plasmin (plasminogen) activity, Tyr nmol/ml		182.6±20.4*	48.6±5.8
Antiplasmin activity, Tyr nmol/ml	220.0±21.6	228.6±20.4	210.8±20.9
Protein, mg/ml		15.3±2.4*	28.6±3.8

* difference statistically significant as compared to blood clot ($p<0.05$)

Discussion

The obtained results show the presence of active thrombin in about 50% of the vein thrombi. It is not found in the blood clot obtained *in vitro*. Thrombin in the vein thrombi evokes blood platelets aggregation and converts fibrinogen into fibrin. Hirudin, as a specific inhibitor of thrombin abolishes its aggregating and coagulating activity in the vein thrombus. Thrombin found in the vein thrombus is bound to fibrin, which makes it resistant to inactivating effect of antithrombin III and heparin [12-15]. Thrombin found in the thrombus may contribute to the vein thrombus enlargement. Fibrin bound thrombin may also evoke rethrombosis observed after thrombolytic treatment of thrombi. Occurrence of rethrombosis was observed after thrombolytic treatment of coronary vessels thrombosis [16,17]. So, the simultaneous treatment with thrombolytic drugs and direct thrombin inhibitors, such as hirudin or synthetic inhibitors is reasonable [18].

The high activity of tissue factor and high antiheparin activity of the vein thrombi promote prothrombin activation and active thrombin formation. Migrating cells such as macrophages, granulocytes and blood platelets are the most probable source of the tissue factor activity and antiheparin factors [19,20].

Increased coagulative activity of the vein thrombi is balanced to some extent by marked activity of plasminogen activators, high activity of plasmin (plasminogen), as well as absence of antiplasmins.

Conclusions

Vein thrombus is characterized by high activity of tissue factor, high antiheparin activity and presence of active thrombin.

High coagulative potential of vein thrombus is balanced to a certain grade by high fibrinolytic potential: high activity of plasminogen activators and high activity of plasmin (plasminogen), as well as absence of antiplasmins activity.

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The use of parotid gland activity analysis in patients with gastro-esophageal reflux disease (GERD) and bulimia nervosa

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Abstract

Purpose: In patients with vomiting or reflux episodes, specific pathognomic signs may occur in the oral cavity. The significance of salivary gland activity in this type of disorder is a matter of dispute. The purpose of this study was to evaluate the parotid gland activity of patients with bulimic type eating disorder (group B) compared with patients affected by gastro-esophageal reflux (GERD) (group A) and healthy control subjects (group C).

Material and methods: Parotid saliva was collected during the clinical examination by means of a modified Lashley cap under unstimulated and stimulated conditions and the flow rate was determined. The concentrations of Na^+, K^+, Ca^+ (mmol/l) were determined by a colorimetric photometry method (Effox 5053, Eppendorf, Germany). Buffering capacity as a concentration of bicarbonates (mmol/l) and the pH, were measured by an automatic ion-selective electrode (ABL TM 520, Radiometer, Denmark). For the statistical analyses Kruskal Wallis one way ANOVA on Ranks with Dunn's method all pairwise multiple comparisons procedures were used with significance set at $p \leq 0.05$.

Results: The results showed that the flow rates in the subjects in group A and B were significantly lower than in the controls. There were also significant differences in the concentration of sodium, with the lowest level in group B, and calcium where the highest level occurred in group A.

Conclusions: Since patients may deny frequent vomiting (bulimia) or are unaware of the reflux (GERD) the changes in electrolyte levels revealed by this study appear to be of use in the diagnosis of these conditions.

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Key words: bulimia nervosa, gastro-esophageal reflux disease (GERD), parotid saliva flow, salivary electrolytes.

Introduction

In the mouth one of the intrinsic factors which leads to a reduction in salivary pH and tooth erosion is gastric acid which has a pH of 1-1.5 [1]. Gastric acid is one of the most important factors in the etiology of erosion in developed countries [2]. Gastro-esophegeal reflux disease (GERD) is the term used to describe a number of conditions where the gastric contents leak into the lower esophagus. Similar conditions apply in conditions such as psychosomatic conditions, eating disorders and alcoholism [3].

The American Psychiatric Association states that bulimic patients regularly binge and purge to keep control of their body weight, "the purging type of bulimia nervosa", or have periods of food restriction and strenuous physical exercise ("the non purging type of bulimia nervosa") [4]. Hoek and Hoeken [5] reported that the incidence of bulimia nervosa in woman is 29: 100000 and in men 1:100000 of those aged between 18 and 29. Epidemiological studies on the incidence of bulimia nervosa, conducted in the United States and Europe, show an increase over the last 40 years [6].

Gastro-esophageal reflux disease is common, and affects between 5-50% people in western countries [7]. Gastro-esophageal reflux is the movement of stomach juices upwards through the lower esophageal sphincter. In healthy individuals small amounts of gastric juice often reflux into the esophagus after eating and may be associated with belching. If the clearance mechanisms cannot return the reflux to the stomach the symptoms become chronic. The reason for this is failure of the lower esophageal sphincter [8, 9]. Two of the constituents of the gastric juice – hydrochloric acid and pepsin are implicated in the pathology of reflux because of their potential to damage the esophageal mucosa. Such a reflux also causes dental erosion and probably changes in saliva [10-12].

Figure 1. **Flow rates of parotid saliva (ml/min) in each group of subjects. 1 – unstimulated saliva, 2 – 3% citric acid saliva stimulated, 3 – stimulated by mastication saliva**

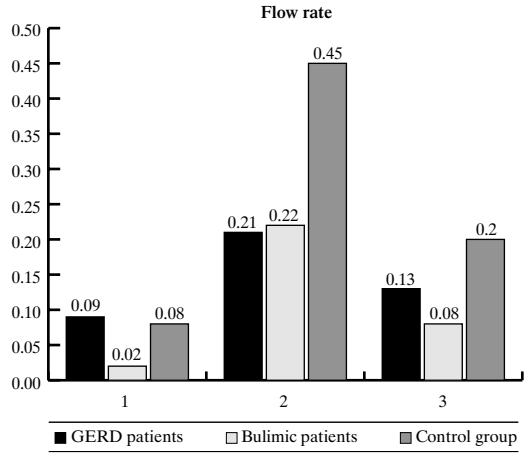
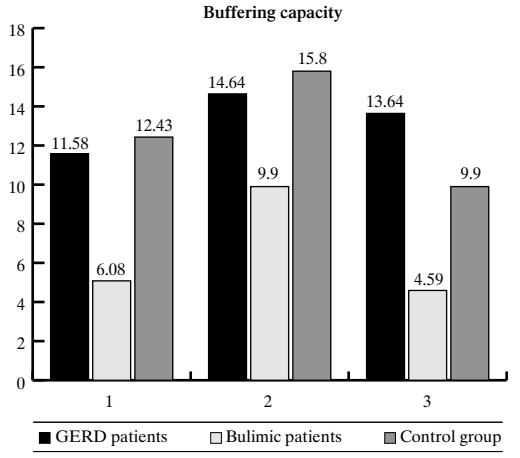


Figure 2. **Mean concentration of bicarbonates (mmol/l) in parotid saliva. 1 – unstimulated saliva, 2 – 3% citric acid saliva stimulated, 3 – stimulated by mastication saliva**



The role of saliva as a neutralizing factor has been reported in a previous research [11]. On the tooth surface, both the formation of the pellicle and a phosphate and calcium saturated condition may protect the teeth from acid. Bicarbonate ions are responsible for the buffering capacity of saliva due to their ability to rise with an increasing flow rate. An explanation for the reduction in both pH and flow rate requires parotid gland activity studies. Because of the changes in oral health which occur in patients with GERD and eating disorders there is a significant need to determine parotid gland efficiency and the nature of changes in the saliva of patients with these conditions.

Material and methods

The purpose of this study was to compare the flow rates and inorganic content of the parotid saliva of 3 different groups, namely 18 gastro-esophageal disease patients (group A), 33 bulimics (group B), and 51 healthy controls (group C). The ethical committee of Poznan University of Medical Sciences granted its approval for this study. The nature of the investigation was explained to the 102 participants, all of whom signed a consent form. The patients in group A, with a confirmed diagnosis of GERD, were referred to the University's Clinical Surgery Department. The mean age of this group was 35±3.2 years, with a mean onset of gastric reflux having occurred 4±2.1 years previously.

The patients in group B, with a confirmed diagnosis of bulimia nervosa of the purging type according to DSM IV (American Psychiatric Association 1994) criteria, were referred to the University's Clinical Psychiatric Department. The mean age of this group was 21.2±3.2 years, with a mean onset of eating disorder having occurred 3.5±2.4 years previously. The mean frequency of binge-purging was 2.0/day. The fifty-one female, age-matched control subjects from group C all denied any history of an eating disorder or gastro-esophageal reflux. Other control selection criteria included: good health, not pregnant, no medication being taken (birth control agents excluded) and no tobacco use. These controls had a mean age of 25.5±4.6 years.

Parotid saliva was collected under both unstimulated and stimulated conditions. All individuals were instructed to refrain from eating or drinking for 1 hour prior to saliva testing. All the saliva collections were performed between 9.00 a.m. and noon and collected by the same examiner. Parotid saliva was collected by a modified Lashley cap placed over Stensen's duct under three different salivary flow conditions: after 15 min rest, physiologically stimulated using 3% citric acid applied to the tongue at 30 s interval and finally when stimulated by the mastication of wax tablets for 5 min. The secretion rate was calculated and recorded in ml/minute. The concentration of the various inorganic components, such as sodium, potassium and calcium (mmol/l) was determined by a colorimetric photometry method (Effox 5053, Eppendorf, Germany). An automatic ion-selective electrode was used for pH determination and bicarbonate concentration (mmol/l) (ABL TM 520, Radiometer, Denmark). Flow rate estimation and electrolyte analysis were performed within 2 hours of saliva collection.

During the clinical examination a level of toothwear was measured using a Smith and Knight Tooth Wear Index [3].

For the statistical analyses Kruskal Wallis one way Anova on Ranks with Dunn's method all pairwise multiple comparison procedures were used. The significance level was set at $p \leq 0.05$.

Results

The results of parotid saliva analyses of the subjects are presented in *Fig. 1* to *7* and *Tab. 1* and *2a*, *b*. Evaluation of parotid secretion showed that in group B the flow rates were significantly lower than in the group A and control group C subjects at rest and under stimulation (*Fig. 1*), 40% of the subjects in group B had unstimulated salivary flow rates <0.01 ml/min. The results present statistically significant differences for pH in the unstimulated saliva between the bulimic group B and control group C

Figure 3. **Mean pH of parotid saliva for all groups. 1 – unstimulated saliva, 2 – 3% citric acid saliva stimulated, 3 – saliva stimulated by mastication**

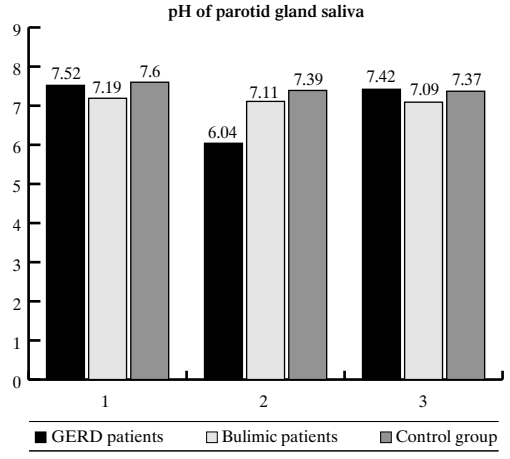


Figure 4. **Mean sodium concentration in parotid saliva (mmol/l). 1 – unstimulated saliva, 2 – 3% citric acid saliva stimulated, 3 – saliva stimulated by mastication**

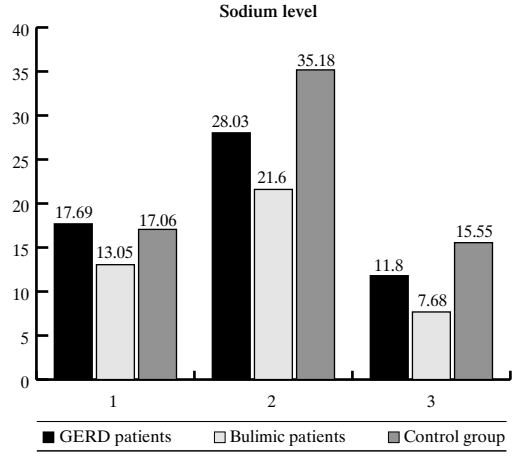


Figure 5. **Mean potassium concentration in parotid saliva (mmol/l). 1 – unstimulated saliva, 2 – 3% citric acid saliva stimulated, 3 – saliva stimulated by mastication**

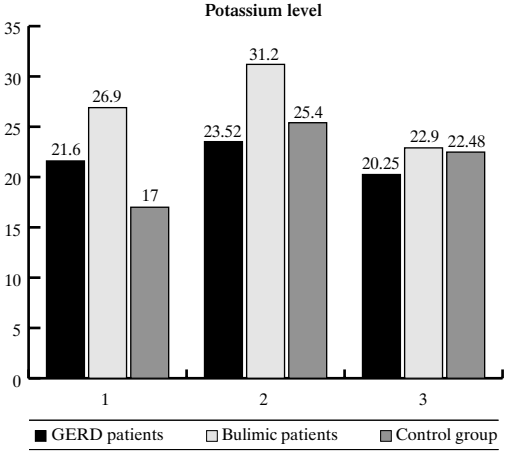


Figure 6. **Mean calcium concentration in parotid saliva (mmol/l). 1 – unstimulated saliva, 2 – 3% citric acid saliva stimulated, 3 – saliva stimulated by mastication**

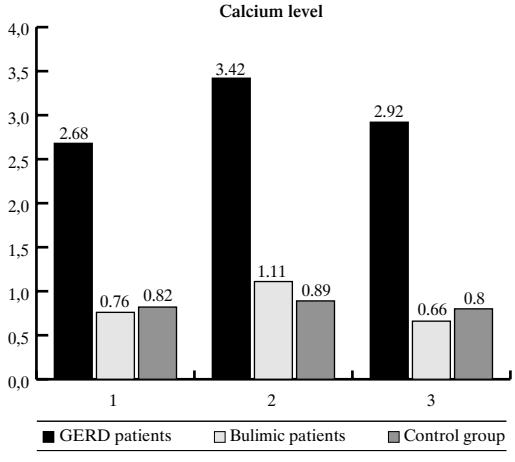
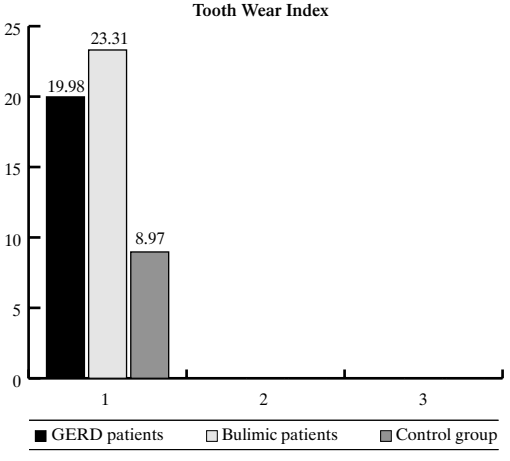


Figure 7. **Mean level of tooth wear in each group measured by the Smith and Knight Tooth Wear Index (%)**



and the bicarbonate level indicating differences between both groups (*Fig. 2*, *3*). Parotid sodium levels increased after stimulation in all three groups but the increase was lowest in the bulimics. A significant difference in sodium levels occurred between groups B and C after chemical stimulation with 3% citric acid (*Fig. 4*). The concentrations of potassium and calcium increased with parotid gland stimulation (*Fig. 5*, *6*). There were significant differences between all groups, especially in the calcium level in groups A and C after chemical stimulation, with the highest concentration in the GERD group A. All the experimental groups (A and B) had significantly more abnormal toothwear than the controls, with the differences being most marked in the bulimic group (*Fig. 7*).

Discussion

The quantity of parotid saliva in bulimics was reduced under both unstimulated and stimulated conditions. Most stud-

***Table 1*. Unstimulated flow rate (ml/min), pH, bicarbonates ions and sodium, potassium, calcium concentrations of parotid saliva (mmol/L). Results are expressed as the mean, S.D. and P value (ns – not significant)**

Saliva data	Group A	Group B	Group C	Comparison	Dunn's multiple comparisons test mean rank difference	P value
salivary flow (ml/min)	0.09±0.06	0.02±0.01	0.08±0.05	A vs B	41.71	***P<0.001
				A vs C	3.21	ns P>0.05
				B vs C	-38.49	***P<0.001
pH	7.5±0.3	7.2±0.7	7.6±0.5	A vs B	10.87	ns P>0.05
				A vs C	-6.54	ns P>0.05
				B vs C	17.42	* P<0.05
bicarbonates ions (mmol/L)	11.58±6.9	6.08±6.1	12.4±11.2	A vs B	60.57	ns P>0.05
				A vs C	8.5	ns P>0.05
				B vs C	52.07	ns P>0.05
sodium concentration (mmol/L)	17.7±12.3	13.05±8.7	17.06±13.7	A vs B	20.67	ns P>0.05
				A vs C	6.08	ns P>0.05
				B vs C	14.59	ns P>0.05
potassium concentration (mmol/L)	21.6±6.4	26.9±8.3	25±5.6	A vs B	-67.6	ns P>0.05
				A vs C	-35.53	ns P>0.05
				B vs C	-25.14	ns P>0.05
calcium concentration (mmol/L)	2.7±1.3	0.8±0.2	0.8±0.3	A vs B	85.64	* P<0.05
				A vs C	86.31	** P<0.01
				B vs C	-0.67	ns P>0.05

***Table 2a*. Stimulated by 3% citric acid flow rate (ml/min), pH, bicarbonates ions and sodium, potassium, calcium concentrations of parotid saliva (mmol/L). Results are expressed as the mean, S.D. and P value (ns – not significant)**

Saliva data	Group A	Group B	Group C	Comparison	Dunn's multiple comparisons test mean rank difference	P value
salivary flow (ml/min)	0.2±0.1	0.2±0.1	0.4±0.2	A vs B	-31.8	*** P<0.001
				A vs C	-4.7	ns P>0.05
				B vs C	-27.05	*** P<0.001
pH	6.04±2.8	7.1±0.7	7.4±0.3	A vs B	-1.4	ns P>0.05
				A vs C	-14.4	ns P>0.05
				B vs C	13.0	ns P>0.05
bicarbonates ions (mmol/L)	14.6±17.5	9.9±9.9	15.8±8.8	A vs B	10.4	ns P>0.05
				A vs C	-40.03	ns P>0.05
				B vs C	50.5	ns P>0.05
sodium concentration (mmol/L)	28.03±9.3	21.6±6.4	35.2±2.4	A vs B	21.1	ns P>0.05
				A vs C	-22.4	ns P>0.05
				B vs C	43.5	* P<0.05
potassium concentration (mmol/L)	23.5±7.7	31.2±8.3	25.4±6.5	A vs B	-68.2	ns P>0.05
				A vs C	-14.9	ns P>0.05
				B vs C	-53.2	ns P>0.05
calcium concentration (mmol/L)	3.4±1.4	1.1±1.8	0.9±0.3	A vs B	94.52	** P<0.01
				A vs C	101.5	*** P<0.001
				B vs C	-6.97	ns P>0.05

ies concerning salivary gland activity in bulimics have reported hyposalivation of different degrees [13-15], except for that reported by Howat et al. [16]. Unfortunately, these authors only examined 11 patients with bulimia and measured the pH, without measuring unstimulated salivary flow.

One possible explanation for the hyposalivation and glandular swelling, which can accompany bulimia could be morphological gland changes caused by inflammation. However, histological investigations have revealed only fatty infiltration and fibrosis, without inflammatory changes [17]. Another explanation for hyposalivation relates to the frequent vomiting or gastric reflux in bulimics which can cause reduced salivary gland output. Other studies have found that the composition of saliva may be changed in bulimic subjects because of dehydration. To support this hypothesis Ship and Fisher [18] conducted an investigation in healthy adults abstaining from eating and

Table 2b. **Stimulated by mastication flow rate (ml/min), pH, bicarbonates ions and sodium, potassium, calcium concentrations of parotid saliva (mmol/L). Results are expressed as the mean, S.D. and P value (ns – not significant)**

Saliva data	Group A	Group B	Group C	Comparison	Dunn's multiple comparisons test mean rank difference	P value
salivary flow (ml/min)	0.1±0.07	0.08±0.02	0.2±0.1	A vs B	20.2	* P<0.05
				A vs C	-15.6	ns P>0.05
				B vs C	35.8	*** P<0.001
pH	7.4±0.4	7.1±0.4	7.4±0.4	A vs B	16.7	ns P>0.05
				A vs C	2.7	ns P>0.05
				B vs C	14.0	ns P>0.05
bicarbonates ions (mmol/L)	13.3±14.7	4.6±3.8	9.9±8.3	A vs B	63.5	ns P>0.05
				A vs C	11.3	ns P>0.05
				B vs C	52.1	ns P>0.05
sodium concentration (mmol/L)	11.8±9.4	7.7±5.1	15.5±11.1	A vs B	23.8	ns P>0.05
				A vs C	-6.0	ns P>0.05
				B vs C	29.8	ns P>0.05
potassium concentration (mmol/L)	20.2±7.3	22.9±9.7	22.5±6.3	A vs B	-32.7	ns P>0.05
				A vs C	-16.9	ns P>0.05
				B vs C	-15.8	ns P>0.05
calcium concentration (mmol/L)	2.9±1.6	0.6±0.3	0.8±0.3	A vs B	115.6	*** P<0.001
				A vs C	104.2	*** P<0.001
				B vs C	11.4	ns P>0.05

drinking for 24 hours and stated that reduced levels of hydration may cause diminished salivary output.

Another possible explanation lies in an electrolyte imbalance resulting from the changes mainly observed in the sodium and calcium levels. Out of all the inorganic components investigated in this study, the most significant statistical differences were found in the sodium levels. Normally, the sodium level increases with the salivary flow rate [19], so the lack of such an increase in the level of this element in bulimic subjects can be additional confirmation of reduced secretion or hyposalivation. Potassium is more independent of the flow rate and is more sensitive to the inflammatory process, due to a breakdown of salivary-blood barriers. The absence of a significant change in the potassium concentration agrees with previous histological studies confirming a non-inflammatory process in vomiting-associated glands [20-22]. A decrease in both these components in the group B, lower salivary flow rate and sodium concentration, suggest that the function of the parotid glands is more affected than that of the other salivary glands. This assumption supports previous studies concerning patients with primary Sjogren's syndrome, after head and neck radiotherapy or neuroleptic treatment. All these hyposalivation groups tended to have an imbalance in salivary sodium concentration [23,24].

The association between vomiting and erosive toothwear was not linearly proven. Reviews in the dental literature showed an increase of erosion with the increase of the frequency of vomiting and gastric reflux. There are also studies that reported no difference in the level of erosion between those who vomited more or less frequently [25,26]. The higher prevalence of erosive toothwear in bulimic patients than in GERD patients may be explained by the fact that the gastric content is not always aspirated to the oral cavity. The regurgitation in GERD patients occurs many times during day but the amount of acidic content is lower comparing with bulimic patients. It is also relevant that among GERD patients the regurgitation may be limited only to esophagus and respiratory system. However, the changes in salivary secretion, electrolyte imbalance and low bicarbonate level may act as a co-factor in erosion.

Conclusions

1. Hyposalivation and lower sodium and calcium levels were found in both Group A (GERD) and Group B (bulimic) subjects which support previous findings of hyposalivation and electrolyte imbalance in such patients.

According to our data bulimic patients have reduced a lover salivary flow from the parotid glands both at rest and under stimulation. It is assumed that this electrolyte imbalance and hyposalivation in group B could account for the greater tooth wear index in this type of patient.

2. According to the results of salivary flow rate is an unreliable indicator of bulimia nervosa and reflux disease. The change of electrolyte level in stimulated saliva could be a more reliable confirmation of symptoms in eating disorder and GERD.

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Plasma fibrinogen concentration in pediatric patients treated with an elimination diet based on soy proteins and casein hydrolyzate

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Abstract

Purpose: Fibrinogen is one of the most discussed new risk factors of atherosclerosis. The aim of the study was to assess the relationship between fibrinogen concentration and classic risk markers of atherosclerosis in a group of children aged from 2 to 6 with or without a family history of circulatory system diseases (FHCAD) (American Academy of Pediatrics – AAP criteria). The study also considered the impact of allergies/food intolerance treatment with elimination diets on the concentration of atherosclerosis markers specially fibrinogen.

Material and methods: Inclusion criteria: a) family history of early occurrence of circulatory system diseases (FHCAD+) according to AAP standards; b) the type and duration of elimination diet continued in infancy and early childhood. 134 of 388 children were included in the investigation.

Results: The analysis of data relating to the so-called classic biochemical risk factors of atherosclerosis (total cholesterol – TC, HDL, LDL, triglycerides, glucose) did not reveal any differences between the tested groups. It was found that in the FHCAD+ group the concentration of fibrinogen was statistically higher than in the group with a negative family history. It was discovered that the type of elimination diet had no effect on fibrinogen level in the FHCAD+ group. In the group of children with negative family history the concentration of fibrinogen was statistically lower in the group on casein hydrolysate than in children treated with soy formula.

Conclusions: The initial interview in pediatrics should include information on the patient's family history of atherosclerosis. In case of a positive family history, fibrinogen, as one of atherosclerosis risk factors, should be monitored.

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Introduction

Fibrinogen belongs to a group of new risk factors in atherosclerosis [1-5], being a biochemical marker of inflammatory status and increased procoagulative readiness of the plasma. Fibrinogen is a 340 kDa glycoprotein synthesized mainly in liver. It consists of four different polypeptide chains, coded by genes located on chromosome 4 [6].

Blood plasma contains 80-90% of fibrinogen. Its concentration ranges from 200 to 400 mg/dl (2-4 g/l). Fibrinogen half-time in the circulatory system is 3-6 days, and its synthesis depends on concentration of its degradation products in plasma and concentration of various cytokines produced by stimulated macrophages [7].

The impact of acute or chronic hyperfibrinogenemia on the development of atherosclerosis may be explained by various mechanisms. Fibrinogen infiltrates vascular walls, causing an increased platelet aggregation and the development of mural thrombi. Another consequence is the rheological effect, i.e. the relationship between high concentration of fibrinogen and blood viscosity, as fibrinogen stimulates migration and proliferation of smooth muscle cells [8].

The results of the most significant population studies on the relationship between the concentration of fibrinogen and the development of atherosclerotic changes are presented in *Tab. 1*.

The analysis of these results has shown that fibrinogen is an independent risk factor for atherosclerosis [8,9]. It is worth noting that increased fibrinogen concentration also occurs in asymptomatic patients.

Higher fibrinogen concentration is related to age, high LDL cholesterol and triglycerides concentration, low concentration of HDL cholesterol, obesity, smoking, and lack of physical activity [10].

Studies carried on adult population confirmed that the predictive value of fibrinogen is comparable to the predictive value of high cholesterol concentration [11-14]. So far we do not have

Table 1. **Chosen population research aimed at determining the concentration of fibrinogen**

Type of test	Number of people tested	Age of people tested	Length of test	Conclusions
Northwick Park Heart Study [8]	1 510 men	40-64	10 years	A significant link was confirmed between the concentration of fibrinogen and the mortality caused by circulatory system diseases; this link was independent of other risk factors and stronger than the link with the total cholesterol concentration. About a half of coronary incidents involved patients with the fibrinogen concentration in the top terce.
Caerphilly/Speedwell Study [24]	4 700 men	45-64	3.2-5.1 years	Fibrinogen as an independent risk factor in ischemic heart disease has predictive quality comparable to the total cholesterol concentration, blood pressure and BMI.
Framingham Study [25]	554 men 761 women	47-79	12 years	Fibrinogen as an independent risk factor for the ischemic heart disease and the cerebral stroke.
PROCAM [26]	1 674 men	40-65	2 years	The frequency of myocardial infarction in the tested group was 2.4 times greater for patients with fibrinogen concentration in the top terce than for those whose fibrinogen was in the bottom terce.
GRIPS Study [27]	5 239 men	40-60	5 years	A significant link was found between the fibrinogen concentration and the occurrence of myocardial infarction. The order of the predictive value: LDL, a positive family history, Lp a, HDL, fibrinogen, age, smoking, glucose, blood pressure.

enough data on the relationship between fibrinogen and the development of atherosclerotic process in pediatric population.

In one of few published studies conducted on the developmental age population Torbus-Lisiecka and her co-workers found that children's concentration of triglicerides and fibrynolysis activity are strongly determined by parental lipids and haemostasis parameters in children with positive family history of hypertriglyceridemia [15]. Similar findings were described by the same author in a study on children with one of parents suffering from ischemic cerebral stroke [16].

Głowińska and her co-workers evaluated the concentration of fibrinogen in a group of children aged from 4 to 20 years with a positive family history of circulatory system diseases [17]. Children included in the study suffered from arterial hypertension, diabetes, and/or obesity. The results didn't show statistically significant differences among the evaluated groups of children.

The correlation between fibrinogen concentration and atherosclerosis in children still remains to be evaluated. Another open question is the influence of food proteins on fibrinogen concentration.

The available data (Medline, Current Contents) seems to lack research on the impact of food proteins on haemostasis, including fibrinogen, in the pediatric populations.

With this in view, we wanted to assess the concentration of fibrinogen and to evaluate the classic risks of atherosclerosis in a group of children aged from 2 to 6 with or without family history of circulatory system diseases (AAP criteria). We have also studied the influence of treatment of allergies/food intolerance with elimination diets on the concentration of the atherosclerotic process markers, mainly fibrinogen. We wanted to test two hypotheses:

1. Fibrinogen level in the plasma of children with a positive family history pointing to the risk of circulatory system diseases does not differ from the level noted in children with a negative family history.

2. Fibrinogen plasma level in children treated with elimination diet based on soy protein in infancy and early childhood is comparable to the level observed in children treated with casein hydrolysate-based formulas. An alternative hypothesis assumes a statistically significant difference in the concentration of fibrinogen in the above-mentioned groups. The study was designed as a clinical-control investigation.

Material and methods

The research, conducted according to the questionnaire method, involved 2 627 children from the north-east of Poland. We adopted strict criteria of including or excluding children from the study groups.

Inclusion criteria into the study group:

1. Type and duration of elimination diet applied in infancy and early childhood. The dietetic treatment has been recommended to children with diagnosed allergy/food intolerance. The group of children on therapy was limited to those with only one substance replacing milk in the diet (either soya or casein hydrolysate formula), with the diet introduced not later than in sixth month of life and continued for at least 12 months. The children who, at any time, have been treated with both types of substances were not included in the study;

2. Family history of early (before 55 year of life) occurrence of circulatory system diseases. A closer and more detailed look into the family history was then aimed at finding the frequency and duration of various medical conditions among members of the family, back to the second generation. They included arterial hypertension, coronary heart disease, atherosclerosis, diabetes, obesity, and high cholesterol level. The children from families in which these medical conditions occurred before 55 years of age were included in the group with a family history pointing to the risk of circulatory system diseases, according to the standards of the American Academy of Pediatrics [18];

3. Children's age: 2-6 years – according to the conducted research at the age of 2 lipid metabolism profile becomes stabilized;

Table 2. **The characteristics of the tested groups**

Group	Diet followed in infancy and early childhood	Family history of circulatory system diseases	The number of children tested	Age [in years] mean (SD)	BMI [kg/m²] mean (SD)	The month of life in which the diet started mean (SD)	How long the diet was followed (number of months) mean (SD)
1	soya protein	+ positive	24	5.66 (1.10)	15.78 (2.16)	4.08 (1.86)	28.21 (12.12)
2	soya protein	- negative	25	5.09 (1.12)	15.31 (1.69)	4.80 (1.12)	30.76 (15.52)
3	casein hydrolysate	+ positive	25	5.15 (1.22)	16.12 (2.29)	3.28 (1.97)	29.28 (9.13)
4	casein hydrolysate	- negative	18	5.03 (1.20)	15.73 (1.92)	3.61 (1.85)	27.11 (11.43)
5	normal diet	+ positive	42	5.47 (1.26)	15.95 (1.52)		

Table 3. **Average values of biochemical parameters for the tested groups of children**

	Group 1		Group 2		Group 3		Group 4		Group 5	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total cholesterol [mmol/l]	4.49	0.78	4.16	0.57	4.48	0.79	4.38	0.62	4.54	0.55
LDL Cholesteol [mmol/l]	2.66	0.64	2.62	0.53	2.86	0.67	2.55	0.65	2.69	0.55
HDL Cholesterol [mmol/l]	1.49	0.38	1.20	0.31	1.26	0.22	1.48	0.50	1.44	0.30
Triglicerides	0.75	0.41	0.77	0.34	0.72	0.19	0.77	0.25	0.81	0.33
Fibrinogen [mg/dl]	277.8	50.03	270.7	49.94	284.2	59.83	239.8[a]	46.12	273.9	62.91
Glucose [mmol/l]	4.64	0.41	4.44	0.41	4.49	0.52	4.52	0.57	4.56	0.51

[a] $p<0.05$ vs group 1,2,3 i 5 (Mann-Whitney U-test)

4. The consent of the patients' parents for participation in the study.

Exclusion criteria from the study group:

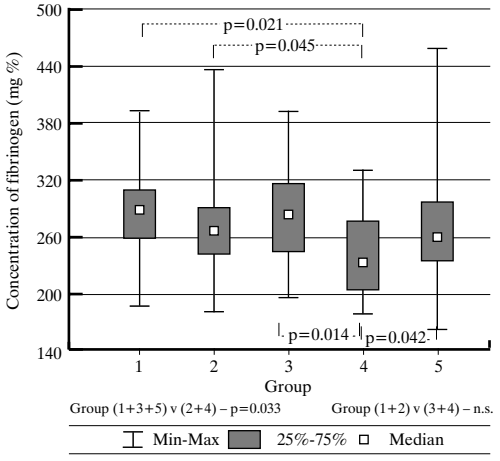
1. Suspected hypertension (diastolic or systolic pressure above 97 centile in two subsequent measurements);
2. Obesity – body weight above 97 centile;
3. History of chronic diseases
   a. glomerulonephritis
   b. chronic bile ducts and liver diseases with and without cholestasis
   c. diabetes
   d. systemic lupus;
4. Infection on the day of inclusion into the study or during the previous 3 weeks;
5. Oral administration of glycocorticosteroids.

The quantity and quality of non-dairy products was similar in all tested groups of children. All children were assign to the study groups and after randomization inside the groups 134 children took part in the tests. *Tab. 2* presents the characteristics of those groups.

Blood samples from all the children were taken from elbow flexure, using minimum stasis. The patients arrived for tests at least 12 hours after the last meal, between 8 and 10 a.m. Blood samples for assessment of lipid metabolism were drawn into glass tubes without any anticoagulant. Routine laboratory methods were used to measure the concentration of total cholesterol, LDL and HDL fractions, as well as of triglycerides and glucose. Blood samples for determination of fibrinogen concentration were taken into test tubes containing 3.8% sodium citrate in the amount allowing to obtain 9:1 blood to anticoagulant ratio. The material was spun at 4°C for 30 minutes. Fibrinogen concentration was measured by Clauss method [19].

The obtained results did not meet the criteria of standard distribution. Therefore they were submitted to statistical analysis using non-parametric Mann-Whitney U-test.

Figure 1. **Mean fibrinogen level in study groups**



The permission to carry out the study was granted by the Bio-Ethical Committee of Białystok Medical University. The parents of the children gave their permission for participation in the test in writing.

Results

Tab. 3 presents the assessed biochemical parameters in the tested groups. The analysis of data linked to the so-called classic risk factors of atherosclerosis, i.e. total concentration of cholesterol and its LDL and HDL fractions, triglycerides and glucose, did not reveal any statistically important differences between the tested groups. A statistically significant difference was found for the concentration of fibrinogen (*Fig. 1*).

It was found that in the group of children with a positive family history of circulatory system diseases the concentration of fibrinogen was statistically higher than in the group with a negative family history (groups 1, 3 and 5, vs 2 and 4; p=0.033).

The analysis of the impact of treatment with an elimination diet on the concentration of fibrinogen in the tested groups revealed that the type of elimination diet did not affect the level of fibrinogen in the group of children with family history of CV diseases. The concentration of fibrinogen was similar in the group of children with a positive family history who have not been treated with an elimination diet.

It is worth noting that among the children with the negative family history the average concentration of fibrinogen was statistically lower in the group on casein hydrolysate diet than the children on soya mixtures (group 4 vs group 2; p=0.045).

Discussion

Our research involved children aged 2 to 6 years. The results show that the concentration of fibrinogen in the group of children with a positive family history of circulatory system diseases of atherosclerotic origin is higher than in children who do not belong to the risk group according to AAP criteria.

It was also found that the concentration of fibrinogen in children from the risk group is not affected by the type of protein used in their nutrition in infancy and early childhood. The research by Pankow et al. [20] was carried on a group of 13 000 men and women aged from 45 to 64, with a positive family history of circulatory system diseases. They evaluated the levels of fibrinogen, VIIc, VIIIc, and von Willbrand factors, protein C, and antithrombin III. The results confirmed that the average concentration of selected parameters, among them classic risk factors of circulatory system diseases, was higher among people with a positive family history [20].

No comparable research has yet been conducted on pediatric population.

The results of published research suggest a positive impact of a soy-based diet on risk factors of atherosclerosis in adults. The research done so far focused on the relationship between the soy-based diet and lipid risk factors of atherosclerosis [21].

According to Anderson and her co-researchers soy protein and soya products reduce the concentration of total cholesterol and its LDL fraction while increasing the concentration of HDL cholesterol [22]. The tests carried among adult patients with hypercholesterolemia revealed that the beneficial effects of soya protein diet are directly correlated to the initial concentration of cholesterol.

Hypercholesterolemia is only one of many risk factors in atherosclerosis. A beneficial, multidirectional effect of soya on the development of atherosclerotic process is well-known [23]. It seems probable that the positive impact of soya also depends on degree of atherosclerotic lesions and the presence of other risk factors in this process.

It is interesting that among the children with negative family history of circulatory system diseases we discovered a significantly lower concentration of fibrinogen in the group whose diet included casein hydrolysates than in the group on soy-based formulas. This result obtained in our study is difficult to interpret, as there is not enough data on the effect of food proteins on haemostasis parameters, including fibrinogen concentration. Solving this problem requires research that could explain the impact of protein amino acid profile on haemostasis parameters. It is possible that the lipid and the amino acid profiles of casein and soya preparations are of some significance. The problem still remains to be solved.

Conclusions

The research conducted on the adults population clearly proves the value of fibrinogen as a risk marker of atherosclerosis. There are still not enough studies on the development of atherosclerotic processes in pediatric population. A frequent occurrence of circulatory system diseases in adult population suggests the necessity of including the patient's family history of atherosclerosis-linked conditions in the pediatric initial interview. In case of a positive family history, fibrinogen could be one of atherosclerosis risk factors to be monitored.

Our study revealed that:

1. In the group of children with a positive family history of circulatory system diseases the concentration of fibrinogen was significantly higher than in the group without such history. The type of diet used did not affect the fibrinogen plasma level in this group of children.
2. A significantly lower concentration of fibrinogen was found in the group of children with a negative family history whose diet included casein hydrolysates in comparison to a similar group treated with soya preparations.

Questions that remain open:

1. Can fibrinogen be considered an independent risk factor of atherosclerotic process in pediatric population?
2. Does the type of diet continued in the infancy and early childhood affect the development of atherosclerotic changes in later life (metabolic imprinting)?

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Empathy in health care providers – validation study of the Polish version of the Jefferson Scale of Empathy

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Abstract

Purpose: Empathy as a crucial component of the interpersonal relationship needs to be measured, especially in helping professions. We designed this study to adapt both "Student" ("S") Version and "Health Professionals" ("HP") Version of the Jefferson Scale of Empathy (JSE) to Polish population.

Material and method: Three instruments were administered to 405 respondents:

– Polish version of the JSE,

– Interpersonal Reactivity Index (IRI) measuring four aspects of empathy (i.e. empathic concern, fantasy, personal distress and perspective taking),

– Emotional Intelligence Scale (EIS).

JSE was applied to physicians, nurses and medical, nursing and midwives students in order to calculate reliability coefficient and other psychometric data. In order to assess validity of the scale, the empathy results were correlated with those obtained by respondents on IRI and EIS.

Results: Cronbach alpha reliability coefficient for "S" version was 0.73, for "HP" version – 0.79, whereas for the entire sample was 0.71. Neither significant differences on empathy scores were found between genders nor among five groups of respondents on JSE. Physicians obtained the highest mean of empathy score (M=113.06), while the lowest was observed in nurses (M=110.12).

Empathy results on JSE correlated significantly with "empathic concern" ($r=0.25$, $p<0.01$) and with "perspective taking" ($r=0.26$, $p<0.01$). Also significant correlation was found between empathy and emotional intelligence.

Conclusions: Despite the lower (but acceptable) reliability coefficient of the Polish JSE in comparison with the original version, the scale proved to be very useful instrument evaluating empathy in health care professionals and students. Further research is needed to identify factors that contribute to changes in psychometric data of the scale.

Key words: empathy, design and methods, tests/interviews-psychometric, other psychological issues research.

Introduction

Meaning of empathy

The easiest way leading to effective care is understanding patient's verbal and emotional behaviours and the attitude of comprehending another person's feelings, emotions and perspective taking. The key instrument improving the therapeutic effectiveness of the clinician-patient relationship is empathy. It's well documented, that the medical care experience is enhanced by effective communication, basis of empathic understanding between clinicians and their patients and that's why the importance of empathy cannot be overemphasized.

What does empathy itself mean and how does it affect doctor (psychologist, nurse, therapist) – patient (client) relationship?

The first researcher, who believed empathy to be one of the most important component of the caregiver – patient relationship, was C. Rogers. He confirmed the meaning of empathy as a factor enhancing therapeutic efficacy. Empathy in Rogers' definition is an accurate understanding of another person's inner experience [1].

Classic definition of empathy by C. Truax describes it as an accurate perceiving of current client's feelings and an attuned way of verbal communicating this understanding to the client. Following his view, many researchers tended to argue, that empathy is a skill and an attitude. In this context it is the ability to communicate one's understanding of the other person' feelings and the reason for his/her feelings [2].

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Empirical data indicates links between empathy and prosocial behaviour, positive attitudes towards old people and confirm its improving influence on health outcomes, better patient compliance, reduction of medical-legal risk and satisfaction of physicians and patients [3].

Components of empathy

The relationship between empathy and helping behaviours occurs in its four components identified by J. Morse et al. [4]:

– emotive – as an ability to experience another person's emotional state,

– moral – as an imperative to be altruistic and to practice empathy,

– cognitive – as an ability to accurate perceiving and understanding another person's point of view,

– behavioural – as communicating one's own understanding of another person's perpective Similarily, C. Patterson also described empathy as a phenomenon stimulating four stages of helping:

• susceptibility of the helper to another person's communicative signals,

• putting helper in the situation of other,

• communicating helper's understanding to the client,

• client's validation of the helper's perception of client's world.

So that, empathy in Patterson's concept consists of four aspects: emotive, cognitive, communicative and relational [5]. However, the Society for General Internal Medicine defines empathy as "the act of correctly acknowledging the emotional state of another without experiencing that state oneself". This definition suggests, that empathy is a combination of two components – intellectual and emotional and, that professional empathy is an cognitive rather than emotional form of understanding other person's behaviour [6].

Primary care clinicians – medical teachers (women – pediatricians and men – internists) who were asked to define empathy, described it as "putting myself in the patient's shoes" and agreed, that empathy consists not only of intellectual understanding and cognitive analysis, but also of emotional connection with the patient. While women – physicians tended to point out emotional component of empathy ("feeling with"), men emphasized the importance of developing empathic behaviours, such as escorting patients, giving direct phone line, prescribing a less expensive medication, etc. [6,7]

On the basis of these outcomes, empathy in patient – care situations may be described as an ability to understand the patient's inner experiences and perspective and to communicate this understanding.

Empathy in health care practicioners

Undoubtedly empathy is a multidimensional form of interaction which involves communication of the health care providers' attitudes of openness and understanding of their patient's world. The empathic behaviours of caregivers are facilitator of trust, coping skills and patient's satisfaction with therapy. Moreover, it protects helpers from burnout and influences their well-being.

It was found, that irrespective of race, nation, country and tradition, those practicioners, who were able to form a warm, friendly relationship with their patients were more effective, than impersonal and formal ones [8].

In the era of high technology and managed health care, the dehumanizing quality of standardized practice discounts the role of empathy and reduces it to a relationship, in which the patient is simply more willing to comply with doctor's recommendations [9]. However, empathic interaction between caregiver and patient means much more than patient's compliance. Its quality influences extremely not only therapeutic outcomes, but, accordingly to La Monica definition of empathy, "[it]… signifies a central focus and feeling with and in the client's world. It involves accurate perception of the client's world by the helper, communication of this understanding to the client, and the client's perception of the helper's understanding" [10].

Though the La Monica's definition of empathy refers to nursing practice, it seems to describe precisely all helper – client relationships.

Empathic understanding is the core of the interaction between physicians (nurses, therapists) and patients. Thus, practicioners to be effective must know how to listen, how to talk to patients and how to communicate their understanding. Listening and empathizing are essential skills when relating to others. Physician's "open" attitude towards patients gives them a feeling of safety, a belief in doctor's abilities and moreover decreases the emotional distance in the doctor–patient interaction.

The procedure of translating original JSE into Polish

Once the permission for translating was obtained, three well-known English language persons (psychologist, physician and sociologist) translated the questionnaire into Polish and then native English speaker (speaking Polish fluently) applied the "forward-backward" procedure. As soon as the provisional version of the Polish JSE has been provided, the questionnaire was further administered to other physicians in order to estimate the comprehensibility of each item. After the consensus by all authors has been obtained, the final version of Polish JSE was developed. The same procedure was repeated with reference to both versions of the scale – Student Version ("S" Version) and Health Professionals Version ("HP" Version)

Two another methods – IRI and EIS used in the study are previously validated instruments, what means that their forward-backward translation and the psychometric data were obtained by the University of Gdansk or the Psychological Tests Laboratory of the Polish Psychological Society.

Aims

This study aimed:

1. To test the psychometric values of Polish version of the JSE,
2. To examine the correlation between Polish version of the JSE and both IRI and EIS,
3. To assess the level of empathy in health care practicioners (including physicians, nurses and students).

Material and method

Procedure

Data were collected in academic year 2003/04. The participants were aware of the study goals and consented to participate

in it. They were instructed not to identify themselves. The only information we asked them to disclose were: gender, age, specialty (in physicians) and seniority (in physicians and nurses). All of the physicians who participated in this study completed the questionnaires either at the end of the residency syllabus (doctors without specialization) or postgraduate education (internists and pediatricians who were obtaining specialization in family medicine). The rest of respondents were administered the questionnaires during under- or postgraduate courses in the field of family medicine.

The measurement of empathy

Empathy as the basis of human relationships was investigated nearly all over the world. In order to estimate its specific components, some instruments were constructed, these were for example: "Interpersonal Reactivity Index" by M. Davis measuring emotional and cognitive empathy, "Hogan Empathy Scale" assessing moral empathy, "Emotional Empathy Scale" by A. Mehrabian and N. Epstein or the "Empathic Understanding of Interpersonal Processes Scale" purposed for nurses [10]. Another instrument measuring empathy is "Empathy Scale" assessing client's perception in the therapeutic relationship with the clinician [9].

Though a great number of studies on empathy in health care professionals have been already done, none of the questionnaires were designed directly to them.

An instrument to measure empathy in health care providers in specific patient care situations was developed by M. Hojat et al. from Jefferson Medical College in Philadelphia. The researchers constructed 20-items scale with three meaningful factors – perspective taking, compassionate care and standing in the patient's shoes, and named it the Jefferson Scale of Empathy (JSE).

JSE was originally developed to measure the orientation of medical students towards physicians empathy (Student or "S" Version). The authors developed also a revised version of the scale to assess empathy in physicians and other health professionals ("HP" Version). The "HP" Version is slightly modified and refers rather to the caregivers' behaviours than to empathic attitudes. Internal consistency reliability (coefficient alpha) on "S" Version was 0.89 for medical students and 0.87 for medical residents. The alpha reliability of the "HP" Version was 0.81. Test-retest reliability was 0.65 with three to four month interval between testing. Both "S" and "HP" Version of the instrument consists of 20 Likert-type items answered on a seven-point scale from 1 – strongly disagree to 7 – strongly agree [11,12]. We asked for consent to translate the questionnaire into Polish and to use it to study empathy in physicians, nurses and students

In order to obtain accurate psychometric data of the Polish JSE and to compare the results with those reported by other researchers, two questionnaires were used additionaly in the study:

– the Interpersonal Reactivity Index by M. Davis (IRI) – a 28-items instrument consisting of four 7-items subscales:

1) Perspective Taking (PT) purposed to measure the individual's dispositional tendency to adopt another person's perspective,

2) Fantasy Scale (FS) intended to provide an indication of an individual's propensity to become imaginatively involved with fictional characters and situations,

3) Empathic Concern (EC) measuring the individual's self-reported tendency to experience feelings of concern for others,

4) Personal Distress (PD) designed to measure the extent to which an individual feels distress as a result of witnessing another's emotional distress.

Each of the 28 items is rated using a five point Likert scale, ranging from 0 – does not describe me well to 4 – describes me well.

IRI is widely used self-report measure of empathy of satisfying reliability and validity. The IRI subscales are regarded as the accurate indicators of social functioning, self-esteem, emotionality, and sensitivity to others and are strongly related to perspective taking, compassionate care and standing in the patient's shoes measuring by JSE.

Internal consistency of IRI ranges from 0.70 to 0.78. 114 significant correlation coefficients (to test validity) between IRI and Wechsler Adult Intelligence Scale, and 466 significant correlations between IRI and Emotional Empathy Scale by A. Mehrabian and N. Epstein were obtained, when exploring relationships between Davis's test and above mentioned methods [13-15].

– The Emotional Intelligence Scale by N. S. Schutte, J. M. Malouff et al. (EIS) – the theoretical basis of the questionnaire is P. Salovey's and J. D. Mayer's model of emotional intelligence (EI). Salovey and Mayer first defined EI as the ability to monitor and regulate one's feelings and those of others and to use feelings to guide thought and action. Emotional intelligence is the ability to perceive emotions, to access and generate emotions so as to assist thought, to understand emotions and emotional knowledge, and to reflectively regulate emotions so as to promote emotional and intellectual growth [16]. In one of their 1990's publications Salovey and Mayer hypothesized that there was a positive relationship between emotional and cognitive empathy and emotional intelligence and that's why the EIS was used in the study. Moreover, emotional intelligence is believed to encompass a variety of social and cognitive functions related to the expression of emotions [16]. The EIS by Schutte, Malouff et al. is a 33-items method answered on a five-point scale from 1 – strongly disagree to 5 – strongly agree. Psychometric studies showed the EIS to have good internal consistency and test-retest reliability. Validation study proved a correlation between EIS and some theoreticaly related constructs, such as: alexithimia, attention to feelings, clarity of feelings, mood repair, optimism and impulse control and showed its predictable value of first-year college grades. EIS scores were significantly higher for females than males and associated with one of the big five personality dimensions – openess to experience [17]. In this study we report the use of JSE ("HP"or "S"-Version), Interpersonal Reactivity Index and Emotional Intelligence Scale in five groups of respondents: physicians, nurses, medical students, obstetrics and nursing students.

Participants

Study participants consisted of 405 respondents (324 women and 81 men) including:

– 118 physicians (95 women, 23 men) with the mean age 38.84 (SD=9.62)

– 76 nurses (women) with the mean age 35.43 (SD=5.72)

Table 1. **Distributions, percentiles and reliability coefficient on the JSE**

Interval	Frequency	Cumulative frequency	Cumulative %
20-40	1	1	0.25
40-60	0	1	0.25
60-80	7	8	1.97
80-100	56	64	15.76
100-120	238	302	74.38
120-140	104	406	100.00
	"S" Version	"HP" Version	Entire sample ("S"&" HP"Version)
Mean	112.40	111.30	111.85
SD	11.40	16.01	13.77
Percentile			
25th	106	104	105
50 th (median)	115	114	115
75th	120	121	121
Possible range	20-140	20-140	20-140
Actual range	67-139	39-137	39-139
Cronbach alpha reliability	0.73	0.77	0.71
Split-half reliability	0.72	0.79	0.72

– 149 medical students (91 women, 58 men) with the mean age 24.73 (SD=1)

– 33 midwives students (women) with the mean age 21.37 (SD=1.16)

– 29 nursing students (women) with the mean age 21.55 (SD=2.08).

The majority of participants are women which is caused by a gender composition. Our sample consisted of family doctors, pediatricians and majors especially dominated by women.

Statistical analysis

All scores were obtained with the use of STATISTICA 6. To investigate the mean scores, medians, standard deviations, minimum and maximum values of the variables, a descriptive analysis were done. A comparison between men's and women's mean empathy scores, among women (physicians, nurses, medical, midwives and nursing students), and between men (physicians and medical students) was done. To compare these means Student t-test or analysis of variance (ANOVA) was used. Levene test was applied to examine the homogeneity of variances. In these cases where the differences between scores were significant, Scheffe post hoc test was done to investigate which groups of participants differ from one another. The split-half reliability and alfa Cronbach reliability coefficient were calculated to assess the reliability of the Polish JSE. The correlations' estimation between JSE and two other methods was considered as preliminary validity test of the empathy scale.

Results

The preliminary psychometric data of the JSE are presented in *Tab. 1*.

Descriptive analysis for the JSE reported in *Tab. 1* showed that the mean empathy score for the entire sample was 111.85 (SD=13.77). The lowest score obtained in the study was 39, whereas the highest was 139. The reliability coefficient calculated by Cronbach alpha for the entire sample was 0.71, for the "S" Version was 0.73 and for the "HP" Version was 0.77. The split-half reliability coefficient for the entire sample was 0.72. Similarly, the split-half reliability coefficient was lower for "S"Version (0.72), than for "HP"Version (0.79). Cronbach alpha for individual items ranged from 0.75 to 0.78 ("HP" Version) and from 0.70 to 0.74 ("S" Version). The mean item scores obtained in the study ranged from 3.08 to 6.59 on the seven-point scale (SD ranged from 1.1 to 2, mode value was 7 on sixteen items). The mean item scores ranged from 3.08 to 6.59 (SD=1.2 – 2) indicate the tendency to be skewed toward the upper end of the scale. The item – total score correlation ranged from 0.10 to 0.60 on the "HP" Version and from 0.14 to 0.62 on the "S" Version.

Number of participants, means, standard deviations of the empathy scores for all groups and summary ANOVA results on the JSE are reported in *Tab. 2*.

Physicians obtained the highest mean empathy scores on JSE, whereas nurses scored the lowest. Results of analysis of variance indicated no significant differences neither between genders (F=1.19, df=1, p=0.28) nor among five groups of respondents (F=0.72, df=4, p=0.58).

Number of participants, means, standard deviations of the empathy scores for all groups and summary ANOVA results on IRI subscales are presented in *Tab. 3*.

The scores on each of the subscales discriminated well between genders. The level of EC (F=24.67, df=1, p=0.00) PD (F=14.62, df=1, p=0.00) and PT (F=3.39, df=1, p=0.06) (the difference nearly significant)) was significantly higher in women than in men. Significant differences were found also when comparing groups of participants. PD and PT were the highest in nurses (PD-F=5.10, df=4, p=0.00, PT-F=2.76, df=4, p=0.02). EC was the highest in nurses too, whereas medical students obtained the highest level of FS. The differences among groups on these subscales were not statistically significant.

Table 2. **Number of participants, means and standard deviations of the JSE ("S" Version or "HP" Version) for genders and individual groups of participants, and summary ANOVA results**

Groups of respondents	JSE		
	N	M (in descending order)	SD
Gender			
Women	324	112.59	12.51
Men	81	110.90	12.16
F		1.19	
df		1	
p		0.28	
Physicians	118	113.06	14.49
Nursing students	29	113.00	12.06
Medical students	150	112.48	10.88
Midwives students	33	112.39	10.34
Nurses	76	110.12	12.87
F		0.72	
df		4	
p		0.58	

N – number of participants, M – mean, SD – standard deviation, F – Fisher test, df – degrees of freedom, p – probability

Table 3. Number of participants, means and standard deviations of the IRI for genders and individual groups of participants, and summary ANOVA results

Group of respondents	EC		FS		PD		PT	
Gender	N	M	SD	M	SD	M	SD	M	SD
Women	324	20.82	3.43	19.36	10.63	17.53	3.66	20.45	3.59
Men	81	18.72	3.38	18.23	4.78	15.82	3.47	19.62	3.81
F		24.67		0.87		14.62		3.39	
df		1		1		1		1	
p		0.00**		0.35		0.00**		0.06∼	
Physicians	118	20.42	3.85	19.40	4.38	16.74	3.30	20.87	3.41
Nurses	76	20.99	3.40	17.91	4.08	18.78	3.40	20.92	3.60
Medical students	149	19.89	3.46	19.65	4.74	16.73	3.75	19.79	3.76
Midwives students	33	20.91	2.73	18.06	4.38	17.57	3.61	19.36	3.31
Nursing students	29	20.55	3.49	19.34	6.02	16.41	4.66	19.76	3.91
F		1.51		0.53		5.10		2.76	
df		4		4		4		4	
p		0.20		0.72		0.00**		0.02*	

EC – empathic concern; FS – fantasy scale; PD – personal distress; PT – perspective taking; N – number of participants; M – mean; SD – standard deviation; F – Fisher test; df – degrees of freedom; $p<0.05$*; $p<0.01$**; p nearly significant ∼

Number of participants, means, standard deviations of the emotional intelligence scores for all groups and summary ANOVA results on EIS are given in *Tab. 4*.

Comparison of emotional intelligence showed significantly higher score in women than in men (F=12.69, df=1, p=0.00). No statistically significant differences were found among individual groups of respondents.

The relationships between empathy scores, IRI and EIS are reported in *Tab. 5* and *6*.

Significant or nearly significant correlations were found between scores on the JSE and relevant measures such as: empathic concern (for physicians, r=0.19, p=0.04; for nursing students, r=0.43, p=0.02), fantasy (for medical students, r=0.22, p=0.06; for midwives students, r=0.55, p=0.00), perspective taking (for nurses, r=0.21, p=0.06; for medical students, r=0.27, p=0.01; for nursing students, r=0.50, p=0.00). JSE correlates significantly with EIS. The correlation coefficients between empathy scores and emotional intelligence were: for physicians, r=0.27, p=0.00; for nurses, r=0.42, p=0.00; for medical students, r=0.31, p=0.00.

The correlations of the total score on the JSE and the scores of the entire sample on IRI and EIS are shown in *Tab. 6*.

Three significant outcomes were noticed when correlating results of the entire sample on empathy scale with IRI and EIS. JSE correlates significantly with two of the IRI subscales: for ECr=0.25, p=0.00 and for PTr=0.26, p=0.00. Significant correlation was also observed between empathy scores and emotional intelligence, r=0.30, p=0.00.

Table 4. **Number of participants, means and standard deviations on the EIS for genders and individual groups of participants, and summary ANOVA results**

Group of respondents		EIS	
Gender	N	M (in descending order)	SD
Women	313	127.29	13.11
Men	80	121.26	14.92
F		12.69	
df		1	
p		0.00**	9.98
Nursing students	29	129.62	12.94
Physicians	109	127.36	13.52
Nurses	72	125.74	12.26
Midwives students	33	125.73	15.12
Medical students	150	124.66	
F		1.42	
df		4	
p		0.34	

N – number of participants; M – mean; SD – standard deviation; F – Fisher test; df – degrees of freedom; p<0.01**

Table 5. **Pearson's correlation coefficients between JSE, IRI and EIS**

	JSE									
	Physician		Nurses		Medical students		Midwives students		Nursing students	
	r	p	r	p	r	p	r	p	r	p
EC	0.19	0.04*	0.17	0.13	0.18	0.11	0.19	0.30	0.43	0.02*
FS	0.04	0.63	0.00	0.99	0.22	0.06∼	0.55	0.00**	0.18	0.36
PD	0.11	0.23	-0.19	0.09	0.11	0.33	0.07	0.71	-0.13	0.49
PT	0.17	0.07	0.21	0.06∼	0.27	0.01*	0.17	0.35	0.50	0.00**
EIS	0.27	0.00*	0.42	0.00**	0.31	0.00**	0.23	0.20	0.19	0.31

r – correlation coefficient; EC – empathic concern; FS – fantasy scale; PD – personal distress; PT – perspective taking; EIS – Emotional Intelligence Scale; p<0.05*; p<0.01**; p nearly significant ∼

Table 6. **Pearson's correlation coefficients between JSE and the scores of all participants on IRI's subscales and EIS**

Scale	JSE	
	r	p
EC	0.25	0.00**
FS	0.08	0.13
PD	-0.02	0.70
PT	0.26	0.00**
EIS	0.30	0.00**

r – correlation coefficient; p<0.01**

Discussion

This study aimed to evaluate the psychometric value of the Polish version of the Jefferson Scale of Empathy – an American instrument measuring empathy in medical students and health care professionals. Any validation study of this instrument was done before in Poland. Psychometric data in support internal consistency showed the Cronbach alpha coefficient 0.73 for "S" Version and 0.77 for "HP" Version, which are within the acceptable range for initial research. Both obtained reliability coefficients are lower than those reported by the authors of the JSE. It makes us to explore the underlying reasons for the results. Two fundamental possibilities should be considered. Firstly – respondents who completed the "HP" Version were primary care physicians (family doctors, internists, pediatricians) and the groups which were administered the "S" Version consisted mostly of final-year medical students. Both the lack of doctors of surgical specialties and age of the students could have become a factors causing our cohort too homogeneous. Secondly – in our opinion, the translation still demands the improvement of linguistic correctness. The studies on these issues are in progress.

Similarly to original version, Polish JSE discriminates between genders to a little extent, although in both research (Polish and American) women's empathy is higher than men's. This and many other research confirm the theory on higher women's sensitivity to others' emotional states and underline women's more accepting attitude toward patients and more related-orientation in the doctor-patient situations [18]. Our studies on empathy in first and final-year medical students proved the gender differences both in the first and the final year of the education [19]. Significant differences between women and men could have been also observed on IRI subscales, especially on "empathic concern", "personal distress" and "per-

spective taking" in the present study. A gender analysis of the empathy results on JSE disclosed lower mean empathy scores in Polish respondents both in women and men (M=112.59 and M=110.9) than scores in American health care providers (M=120.9 and M=119.1) reported by Hojat, Gonella et al. [12]. We suggest two explanations of these results. Firstly, it is possible, that some items of the JSE after they had been translated into Polish lost their accuracy, and secondly – both cultural and curriculum differences between Polish and American health care providers should be taken into consideration. To our knowledge, more humanistic and patient-oriented topics is being taught during undergraduate and postgraduate education in American medical universities.

In spite of still existing defects of the Polish JSE, the results of empathy obtained by the sample correlate highly with IRI and EIS. Though the IRI was developed for a general population in opposite to JSE which was designed directly to health care practitioners and students, two IRI subscales (EC and PT) correlate significantly with the total score of JSE. To our satisfaction these findings are similar to those reported by Hojat et al. [20], who discovered that perspective taking (PT) and empathic concern (EC) are the most relevant to patient care situation and reflect the patient-physicians relationship, while two other factors – fantasy (FS) and personal distress (PD) are attributes less relevant to the patient-physician relationship.

The connection between empathy and emotional intelligence supports the Salovey's and Mayer's theory which assumes emotional intelligence to be a form of social intelligence that involves the ability to monitor one's own and others' feelings and emotions, to discriminate among them, and to use this information to guide one's thinking and action. Empathy skills are those that involve paying attention to other people things like listening, attending to needs and wants of others, and building relationships. Once the emotional intelligence increases, one is more likely to recognize other people's point of view and to satisfy their expectations accurately [17]. In our research women's emotional inteligence is statistically higher than men's.

The relationships between above mentioned methods support the diagnostic value of the Polish version of the JSE as the psychometrically sound instrument.

Realizing that Polish version of the JSE deserves further research attention including linguistic improvement and applying it in more diverse samples, we are deeply satisfied from the possibility to evaluate the level of empathy in health care professionals with specific instrument. In the nearest future we intend to compare student's empathy in the early and final stages of medical undergraduate education and to estimate its level in doctors of "people-oriented" and "technology-oriented" specialties.

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Improvement in the accessibility and organization of services of family physicians in a small town in Poland: a comparison of patient opinions between 1998 and 2002

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Abstract

Purpose: The institution of a family physician was introduced in Poland to improve organization and quality of primary health care. Thus, it seems important to find out how the time factor (4 years) and the organizational changes that took place during that time have affected patients' views on primary health service accessibility.

The aim of the study was to compare patients' opinions on selected aspects of the access and organization of health care provided by family physicians between the years 1998 and 2002.

Material and methods: Two independent surveys conducted in 1998 and 2002 using face-to-face interviews with structured questionnaires. The study was carried out in a small town in Poland. The study group consisted of two samples of patients randomly selected. Altogether 1000 interviews were obtained in survey I (1998) and 1000 from another sample in survey II (2002).

Results: The surveys carried out at a 4-year interval showed that the accessibility of family physician services improved between 1998 and 2002. This was reflected by: more common use of registration by phone and better overall evaluation of the registration system, shorter time spent in the waiting room to see a family physician, making an appointment for a definite hour, better opinion of the visit duration, more frequent use of phone consultations and higher number of home visits.

Conclusions: The results our study show that primary health care reform in Poland has a positive impact on the patients' opinions about access and organization of services of family physicians.

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Key words: primary health care, health services accessibility, outpatients.

Introduction

The Polish health care system, similar to other Central and East European countries, has been transforming from the state run system to less centralized model [1-5]. Since 1990, the government of Poland has introduced a number of reforms in the finance, management and organization of the health sector; a dynamic development of the private sector started, mainly in dental care, ambulatory services, and diagnostic testing. Since 1993, private surgeries and other medical organizations have been able to sign contracts for the provision of services to the persons entitled to care financed from public resources [1]. Important changes have been taking place in the primary health care system. The aim of the reform was to change the system, which used to be centrally governed and based on specialists providing services in primary health care, into a more effective, cheaper one that offers better services. Before the reform, primary health care was provided by multispecialist teams of physicians trained in internal medicine, paediatrics, and gynaecology, and by dentists, nurses, midwives and ancillary support staff. The model was disease and specialists oriented. All health care personnel were state employees and paid on salary-basis. Despite the large number of specialists, access to care in the public sector was often difficult.

Changes in the financing of health care, from a budget-financed system to mandatory health insurance (National Health Found) have promoted the development of a private sector, including primary health care.

The main features of primary health care reform were: (1) implementation of family physicians in 1995 and recognition of family medicine as a specialty with its own under- and post-graduate training programs; (2) introduction of the contracting and remuneration system of primary care doctors together with a list system for family physician; (3) privatization of primary health care facilities with family physicians becoming the owners and employers to other staff.

According to the reform assumptions, the work of family physicians involves a number of prerequisites treated as a whole, namely continuity of care, coordination, health promotion, and care within the family and community setting.

The way in which health care is organized and financed appears to be related to patient satisfaction, especially in outpatient settings [6-7]. International studies show, that patients in different cultures and health care systems may have different views on some aspects of care, but most of them share opinions relating to doctor – patient communication and accessibility of services [8]. Access to care includes being able to make an appointment to see one's physicians in a timely fashion, not having to wait a long time in the physician's office, and being able to speak to him/her on the telephone [9]. Donabedian [10] states that accessibility is "the ease with which person can obtain care". According to Campbell et al. [11] access is one of the principal dimensions of quality of care for individual patients and organizational access is a sub-component of availability. If people are physically able to access a health facility they may still face barriers to accessing care in terms, for example, of the length and availability of appointments.

Patients can assess various accessibility elements, such as doctor – patient relationship, receptionists, appointment system, waiting time, consultation length, telephone services, home visits, distance to cover and others [3,4,7,9,12].

In the current study, we focus on these aspects of access and organization of services which had been frequently criticised by Polish patients in the previous system of primary health care. The most common problem was registration for the visit and long queues in the waiting room [13-15]. Moreover, patients complained that they could not choose their physician [16].

The institution of the family physician in Poland based on the model operating in many Western European countries was an attempt to improve primary health care accessibility and effectiveness. There is little evidence available in the literature of the subject about the impact of the reform of primary health care in former European communist countries on the improvement of the access and organization of services. Furthermore health care reform projects are rarely evaluated systematically [17]. Thus, it seems important to find out how the time factor (4 years) and organizational changes affected patients' opinions in the aspect of medical service accessibility.

The aim of the study was to compare patients' opinions on selected aspects of the access and organization of health care provided by Polish family physicians between the years 1998 and 2002.

The following topics were studied: the system of registration, length of time spent in the waiting room to see a doctor, the appointment system, length of consultation, telephone advice, home visits, problems associated with the use of health care services and overall satisfaction with family physician care.

Material and methods

Setting

The study was carried out in a small town (Giżycko) in Poland, where physicians with specialist training in family medicine were introduced in 1995. Giżycko was choosen to be the study setting for it was one of the first towns in Poland to have a new family physician – based primary health care model instituted. Moreover, the study gained approval of both family physicians and local health care authorities, who wanted to know the patients' opinions about primary health care reform. Giżycko is a small town in the north-eastern region of Poland, with approximately 40 000 inhabitants. In 1998 (survey I), the total number of patients of family physicians amounted to 36 281 and 15 family physicians worked in the studied area, each having the list of patients. They signed contracts with the local government and were financed from the provincial budget.

In 2002 (survey II), in the same area, the total number of patients of family physicians amounted to 35 525 and there were 16 family physicians, each with a list of patients. They signed contracts with the Regional Sickness Fund following the introduction of the universal health insurance administered by Health Insurance Funds.

Both in investigation I and II, the capitation system was used as the basis for service compensation and the amount of financial means depended on the number of registered patients (capitation-based physician payment). Within the capitation fee, family physicians provided health services according to their professional competence.

Sample

The study group included patients randomly chosen from those registered on the patient lists of a doctor and (or) nurse, who visited the doctor's or nurse's centre or were visited at home by them in the preceding week.

In 1998 (survey I), every fourth person (1 023 subjects) was selected from the list of 4 092 eligible patients. Altogether 1 000 interviews were obtained. In 2002 (survey II), every fourth person (1 016 names) was chosen from the list of 4065 eligible patients. As only 988 interviews were obtained, an additional 12 subjects were randomly selected from the list so as to achieve a similar sample size as in survey I.

Questionnaire

A structured questionnaire was devised for the survey. The questionnaire contained questions on the use of health care services, patient demographic and health characteristics, as well as opinion and experiences with family physicians' and family nurses' care.

The study was preceded by a pilot investigation to elucidate whether questions were properly understood and answered. The questionnaire was slightly modified in survey II – certain questions were deleted, but its form was maintained to facilitate comparison.

The information was collected by means of face-to-face interviews. The respondents were interviewed in their homes by trained interviewers.

In this paper we analysed only the results related to access and organization of services of family physician care.

Ethics

The Ethics Committee of Medical University of Białystok approved the study.

Table 1. **Comparison of respondents' characteristics in the two surveys**

	Survey I	Survey II
Characteristics	n=1000 (100%)	n=1000 (100%)
Age (years)		
Under 24	118 (11.8)	100 (10.0)
25-44	391 (39.1)	369 (36.9)
45-64	274 (27.4)	278 (27.8)
65-74	142 (14.2)	155 (15.5)
+75 years	75 (7.5)	98 (9.8)
Sex		
Women	689 (68.9)	689 (68.9)
Men	311 (31.1)	311 (31.1)
Education		
Elementary and lower	325 (32.5)	306 (30.6)
Technical	220 (22.0)	223 (22.3)
Secondary and post-secondary	362 (36.2)	383 (38.3)
University	93 (9.3)	88 (8.8)
Frequency of visits to family physicians in previous 12 months*		
none	24 (2.4)	5 (0.5)
once	51 (5.1)	36 (3.6)
two-three times	198 (19.8)	175 (17.5)
four-five times	232 (23.2)	206 (20.6)
six and more	495 (49.5)	578 (57.8)

* P<0.0001

Analysis

Data were encoded and analysed using a packet Statistica PL v.7.1. Chi-square test was used to assess the correlation between non measurable features and $p<0.05$ was considered statistically significant.

Results

Tab. 1 gives details of the characteristics of patients in survey I and survey II. The sample characteristics in the two surveys were similar. There was no significant difference between the two groups in relation to age, sex and education. Statistically significant differences referred to frequency of visits to family physicians in the previous 12 months (P<0.0001). In survey II, there were less patients who had no visit at the doctor's or visited him/her once, twice, three, four or five times. Those coming to the doctor six or more times were more.

Registration

A comparison of distribution of replies to the question concerning registration mode, between survey I and II, revealed statistically significant differences. In survey I, 8.8% of patients registered by phone, while 4 years later – 41.7%. However, making an appointment for the visit personally is still more common. In survey I, 84.7% made an appointment in this way, while in survey II – 54.2% (*Tab. 2*).

The overall assessment of registration for the visit was based on the choice of 5 categories, from very good (5) to very bad (1). The mean assessment of registration in survey II was higher – 4.25 than in the previous one – 4.16 (P=0.0003).

Table 2. **Registration**

Registration	Survey I	Survey II
	n=1000 (100%)	n=1000 (100%)
By phone*	88 (8.8)	417 (41.7)
Personally*	847 (84.7)	542 (54.2)
By other persons	36 (3.6)	35 (3.5)
Scheduled by physician	2 (0.2)	6 (0.6)
Lack of answer	27 (2.7)	0 (0.0)

*P<0.00001

Table 3. **Length of time waited to see the physicians (time in the waiting room) and appointment**

The waiting time and appointment	Survey I	Survey II
	n=1000 (100%)	n=1000 (100%)
The waiting time		
less than 15 minutes*	323 (32.3)	586 (58.6)
more than 15 minutes, less than 1/2 hour	335 (33.5)	329 (32.9)
more than 1/2 hour, less than an hour*	222 (22.2)	61 (6.1)
more than an hour*	93 (9.3)	21 (2.1)
lack of answer	27 (2.7)	3 (0.3)
*P<0.00001		
Appointment		
Yes	78 (7.8)	503 (50.3)
No	894 (89.4)	495 (49.5)
Lack of answers	27 (2.7)	2 (0.2)

P<0.00001

Waiting time and possibility of making an appointment for the visit, consultation time

It was found that the waiting time for the visit shortened significantly between the surveys. This means that the percentage of subjects waiting longer than half an hour decreased, while the percentage of those waiting shorter than 15 minutes increased (*Tab. 3*).

This may be due to the fact that during survey II more patients visited their physician by appointment. In survey I, only 7.8% of the respondents made an appointment for a definite hour, while an increase to 50.3% was noted in survey II (*Tab. 3*).

Between survey I and II, patients' opinions on the length of their last visit changed significantly. In survey II, patients more frequently considered the last visit duration to be sufficient and fewer patients complained that the visit was too short (*Tab. 4*).

Telephone advice

It was established that in survey II, the number of patients aware of the possibility of telephone consultation increased. The possibility of medical consultation within the working hours was known by 68.1% of the respondents in survey I and by 76% in survey II. The percentage of subjects who had no idea of this form of advice decreased. The difference is statistically significant – P=0.0001 (*Tab. 5*). However, a small percentage of the respondents used this mode of contact with their family physicians in the last month; in survey I – 133 subjects (13.3%), in survey II – 143 (14.3%).

Table 4. **Length of consultation (duration of the last visit at the family doctor's)**

Length of consultation	Survey I	Survey II
	n=1000 (100%)	n=1000 (100%)
Definitely too long and rather too long	9 (0.9)	6 (0.6)
Sufficient	829 (82.9)	911 (91.1)
Rather too short	113 (11.3)	77 (7.7)
Definitely too short	22 (2.2)	5 (0.5)
Lack of answers	27 (2.7)	1 (0.1)

P<0.0001

Table 6. **Is it easier nowadays to get advice at the family doctor's?**

Is it easier to get advice?	Survey I	Survey II
	n=1000 (100%)	n=1000 (100%)
Definitely easier*	322 (32.2)	444 (44.4)
Rather easier	399 (39.9)	392 (39.2)
No change	173 (17.3)	105 (10.5)
Rather more difficult	90 (9.0)	50 (5.0)
Definitely more difficult	16 (1.6)	9 (0.9)

*P<0.00001

Overall satisfaction with telephone advice within the range from very much satisfied to very much dissatisfied was higher in survey II – the percentage of respondents very satisfied increased from 33.1 (survey I) to 64.3 (survey II) (*Tab. 5*).

Distance

In survey II, fewer patients lived in the close vicinity of the family physician practice compared to survey I. In 1998 there were 907 patients (90.7%) living up to 5 kilometres from the practice while in 2002 – 868 such patients (86,8%). In survey II, patients more frequently claimed that the practice location was inconvenient compared to survey I of inconvenient localization of the physician's surgery (28.5% and 21.5% respectively).

Home visits

Over twice as many patients asked for the home visit – within the last month – in survey II – 111 subjects (11.1%) compared to I – 45 (4.5%). Home visit refusal (once) was reported by 14 subjects in survey I and 17 patients in survey II, several times – 9 subjects in survey I and 5 in survey II.

Barriers

The patients were presented a list of nine possible difficulties in the use of services provided by a family physician and were asked to point at one to three of them as the most strenuous. Moreover, the patients could supplement the list with their own statements.

The most commonly reported difficulties referred to:

1. obtaining a referral to a specialist (30.8% – I and 25% – II),
2. having diagnostic tests (30.2% – I and 17.7% – II),
3. too long distance to the family physician practice (23% – I and 31.3% – II),
4. queuing in the waiting room (19.5% – I and 5.7% – II).

Table 5. **Telephone advice**

Telephone advice	Survey I	Survey II
	n=1000 (100%)	n=1000 (100%)
Is there a possibility of telephone consultation?		
Yes	681 (68.1)	760 (76.0)
No	41 (4.1)	43 (4.3)
Do not know	278 (27.8)	197 (19.7)
P=0.0001		
Have you phoned your family doctor for advice? (within the last month)		
Yes	133 (13.3)	143 (14.3)
No	867 (86.7)	857 (85.7)
Not significant		
Satisfaction with telephone advice	133 (100%)	143 (100%)
Very satisfied*	44 (33.1)	92 (64.3)
Rather satisfied	76 (57.1)	38 (26.6)
Difficult to say	6 (4.5)	8 (5.6)
Rather dissatisfied	4 (3.0)	4 (2.8)
Very dissatisfied	3 (2.3)	1 (0.7)

*P<0.00001

Table 7. **Satisfaction with family doctor**

Satisfaction	Survey I	Survey II
	n=1000 (100%)	n=1000 (100%)
Very satisfied	377 (37.7)	350 (35.0)
Rather satisfied*	435 (43.5)	501 (50.1)
Difficult to say	110 (11.0)	98 (9.8)
Rather dissatisfied	59 (5.9)	45 (4.5)
Very dissatisfied	19 (1.9)	6 (0.6)

*P=0.003

As revealed by the above data, the lower percentage of subjects reported difficulties with obtaining a referral to a specialist, having diagnostic tests and queuing in the waiting room. However, more patients complained of a too long distance to the surgery.

The patients' replies to the question "Is it easier nowadays to obtain medical consultation at the primary health care physician's than before?" confirm that the changes in the primary health care are becoming increasingly popular with patients (*Tab. 6*).

Overall satisfaction with the family doctor care was measured on a five-point scale (very satisfied, rather satisfied, difficult to say, rather dissatisfied, very dissatisfied). Comparative analysis show that in survey II, the percentage of respondents rather satisfied with family doctor care increased from 43.5 to 50.1 (P=0.003) (*Tab. 7*).

Discussion

Surveys of patients' opinions concerning primary health care are not new in Poland, e.g. studies by other authors were undertaken in the 60s-70s [16] and 80s [13-15] of the previous century. However, there have been many changes in primary

health care and the views of patients have changed as well. Our findings show that the primary health care reform in Poland has exerted a positive impact on the patients' views on the access and organization of services of family physicians in a small town in Poland. Studies of other authors – using different methodology – also indicate that significant improvement was noticed in consultation availability in the primary health care [18].

In our study, the accessibility of family physician services improved between 1998 and 2002. This was reflected by: more common use of registration by phone and better overall evaluation of the registration system, shorter time spent in the waiting room to see a family physician, making an appointment for a definite hour, better opinion of the visit duration, more frequent use of phone consultations, and higher number of home visits. The appointment system comprising visits at a definite hour and telephone consultations is a new element that builds up the family physician profile in Poland, not earlier encountered in the system of primary health care. In the socialistic system of health care, queuing in the waiting room at the doctor's was the most common objection raised by patients against health service. Over 56.3% of patients reported waiting for the visit as long as over one hour [16]. The appointment system is a major factor determining patient satisfaction with a family physician [4].

In our study, a significant increase in the number of patients who see their physicians by appointment suggests that this form of service provision is highly acceptable.

Telephone consultations are very common in many countries and their accessibility is felt to be a positive aspect of the service [12]. In Poland, this mode of service provision is new and as shown by our study not very popular with patients. In the area involved in the present study, patients seldom call their family physicians to receive medical advice, although for the majority of the interviewes telephone consultations are a well known and accepted form of medical service. There seems to be a need for detailed recommendations and guidelines on telephone organization in practice of family physicians.

The fact that in 2002 (survey II) a smaller percentage of subjects reported problems with obtaining a referral to a specialist or accessory investigations may result from the contract contents. In 2002, referrals to a specialist did not encumber the family physician budget and part of the capitation-based payment was designed for diagnostics. In 1998, the financial means for diagnostics and specialist consultations were included and thus burdened the family physician budget.

It should be noticed that patients' satisfaction with family physicians was high both in study I and II (see *Tab. 7*). This seems to confirm that in quantitative studies, the question concerning patients' satisfaction usually provides approximately 80% of positive responses [19].

The results our study show that primary health care reform in Poland has a positive impact on the patients' opinions about access and organization of services of family physicians.

The improvement in service accessibility revealed in the study may have at least two reasons. Firstly, family physicians have improved organization of services to make their work easier and more effective. This is particularly important in the situation determined by private medical services and competition for the patient. Secondly, patients' habits and expectations of service organization may have been altered throughout the 4-year period.

The primary health care reform in Poland is a continuous process exhibiting certain tendencies, which on one hand result from patients' expectations and are due to legal regulations on the other. Therefore, the changes that occur should be monitored with the involvement of patients. As claimed by experts, patients have become increasingly engaged in the evaluation of care [20]. Therefore, patients' views may be taken into account in service contracts between purchasers and providers. It is particularly important in the situation of market competition among providers, with patient as the main goal.

This study had certain limitations, which should be noted. Firstly, as the research was conducted in a small town, it does not necessarily reflect patients' opinions in other regions of Poland, especially in big cities. Secondly, this study provides only quantitative data on some of the service accessibility components. In depth qualitative methods may be necessary to obtain more valid evidence.

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Differentiation of IgE-dependent and IgE-independent reactions in children with bronchial asthma on the basis of TOP CAST Paediatric Allergen Mix test

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Abstract

Purpose: TOP CAST Paediatric Allergen Mix test is a new cellular *in vitro* test based on evaluation of leukotrienes synthesised by basophils under the influence of specific allergens. The aim of the study was evaluation of applicability of this test as screening examination in diagnosis of atopic asthma in children.

Material and methods: The study was carried out on a group of 30 children (56.7% boys and 43.3% girls) aged 6-15 yrs (mean age 8 years and 9 months, SD=2.1) with diagnosed bronchial asthma. In children qualified for the study clinical symptoms, subject examination as well as functional examination of the respiratory system (obturation with positive reversibility test) confirmed the disease. All the children had skin prick tests performed with the most popular aero- and troph-allergens, which results were expressed (+) according to the Skandinavian scale. In 15 cases asthma had atopic origin: in 11 children – mites were responsible for the contraction of bronchi, in 3 cases – tree-pollens allergens and in 1 case – grass pollens. In 15 next cases non-atopic asthma was diagnosed. The control group consisted of 10 children without clinical manifestations of asthma and negative results of the above tests. Test TOP CAST Paediatric Allergen Mix with mixture of 21 inhalatory and food allergens was performed according to the producer's procedure.

Results: Statistically significant differences of the values of released leukotrienes were noted at allergen concentration of both 100 ng/ml and 10 ng/ml in children with diagnosed atopic asthma compared to those with non-atopic asthma and control group. The sensitivity of TOP CAST Paediatric Allergen Mix test was 80% at both allergen concentrations while the specificity was higher (90%) at the lower concentration. There was also correlation between the number of released leukotriens and IgEc in the examined group of children, however, no statistically significant differences were observed between the concentration of the released leukotrienes and the size of the wheal and the number of positive skin prick tests.

Conclusions:

1. TOP CAST Paediatric Allergen Mix test is a good screening method in differentiation of atopic and non-atopic background of bronchial asthma in children.
2. At the present evaluation stage of this test, it may be applied as complementation of routine tests in allergological practice.

Key words: TOP CAST, leukotrienes, atopic asthma in children.

Introduction

Bronchial asthma is one of the most frequent chronic ailments of the respiratory system in children, which constitutes a serious problem of public health.

Diagnosis of bronchial asthma is based on history, recognition of typical clinical manifestations and additional tests. In most cases functional examination of respiratory system, skin prick tests and total IgE and allergen-specific IgE determination are sufficient for the diagnosis.

However, in some children a complex diagnosis is difficult or impossible (due to age, skin alterations, application of antihistamine drugs) or the results of widely used tests are equivocal. Regarding these, search for new methods applicable widely in diagnosis of allergic ailments, including atopic asthma present in most cases of asthma (80-90%) in population at developmental age, still remains an open issue.

CAST-ELISA (Cellular Allergen Stimulation Test) Method

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is based on measurement of cysteinyle leukotrienes generated by peripheral blood leucocytes under the influence of specific allergens or other factors (drugs, lipopolysaccharides, insect venom, food) [1,2]. In Poland this method was applied in adult patients suspected of allergy to mites, plant pollens, venoms of insects or drugs and in order to monitor specific immunotherapy [3-13]. Our own experience confirms usefulness of this test in diagnosis of asthma stimulated by allergens of home dust mites and pollens in children [14-16]. Therefore we carried on with the work to evaluate applicability of the new TOP CAST Paediatric Allergen Mix test, consisting of 21 inhalatory and food allergens, as screening examination in differentiation of the background of atopic bronchial asthma in children. The possibility of performing a screening examination of a small amount of blood (2 ml) detecting sensitivity to common inhalatory and food allergens would make a valuable diagnostic method in exogenous asthma in children.

The aim of the study was evaluation diagnostic applicability of TOP CAST Paediatric Allergen Mix test in differentiation of IgE-dependant and IgE-independent bronchospastic reactions in children.

Material and methods

The study was carried out on 30 children (17 boys – 56.7% and 13 girls – 43.3%) between 6-15 yrs (mean age 8 years and 9 months, SD=2.1) with diagnosed bronchial asthma. The duration of the disease was 2-8 years (mean 4 years). Diagnosis of bronchial asthma was based on data obtained from: history (characteristic features of the ailment), results of functional examination of the lungs during acute phase of the disease (obturation of the bronchial tree) confirmed by reversibility test (ΔFEV1>15%). In all patients skin prick tests were performed with inhalatory allergens (mites, grass, tree, weed pollens, cat and dog fur, mildew) and food allergens (milk, egg, hen meat, fish, orange, nuts, flour, soy). Skin tests with aero- and troph-allergens were performed with the prick method with Allergopharma kit. Skin reaction was compared to the diameter of the histamine wheal (the result was expressed in Scandinavian scale from 0 to 4+). For the study we qualified patients with skin reaction at least at 3+ or 4+.

In 15 cases (12 boys and 3 girls, mean age 7 years and 8 months, SD=2.5) asthma had atopic origin: in 11 cases the factors triggering asthma attack were mites, in 3 – tree pollens and in 1 – grass pollens. In 15 next cases (5 boys and 10 girls, mean age 7 years and 7 months, SD=1.7) non-atopic asthma was diagnosed (positive reversibility test, negative skin tests).

In all sick children any respiratory tract infections, inborn malformations of the bronchial tree and lung tissue, heart defects, gastroesophageal reflux or immunological insufficiencies were excluded.

The control group consisted of 10 healthy children (4 boys and 6 girls) aged between 6-17 yrs (mean age 10 yrs, SD=3.8) with negative history of allergic diseases or subject examination. Functional examination of the lungs didn't reveal any disorders of bronchi patency, and the results of skin tests were negative with inhalatory or food allergens.

Before CAST-ELISA Paediatric Allergen Mix procedure was applied, for a least 14 days the patients received no medical treatment nor any allergy symptoms were observed. After physical examination excluding any respiratory tract infections, venous blood was taken in the amount of 3 ml per 0.2 mmol of disodium versenate in volumetric proportion 20:1. Then it was mixed with dextran from a Buhlmann Laboratories A.G. kit in proportion 4:1 and incubated in room temperature for 90 minutes. After that the upper phase was collected and centrifuged for 15 minutes with 130 g. The supernatant was drained off and the sediment was mixed with stimulation buffer. The suspension was placed in test tubes, 200 μl in each and treated with 50 μl of specific inhalatory and food allergens in concentration of 100 and 10 ng/ml. To the test tubes labelled as spontaneous generation of leukotrienes we added 50 μl of stimulative buffer. The samples were incubated in 37°C for 40 minutes and centrifuged for 3 minutes at force of 1000 g. The supernatants collected from each test tube were frozen for 14 days. Then the wells coated with leukotriene antibodies were supplemented with 100 μl portions of: non-specific bond standard, zero standard, standard leukotriene solutions in 4 concentrations and supernatants after cells stimulation. To all the wells we also added 50 μl solution of leukotriene conjugated with alkaline phosphatase and 50 μl of leukotriene antibodies solution. The plate was incubated for 24 hours at 4°C, and then after emptying all wells and double-washing, 100 μl of substrate solution was added and incubated for 30 minutes at 20°C. Then 100 μl of inhibitor solution was applied, mixed and the absorbency was measured at 405 nm. Leukotriene concentration was recorded in relation to the standard curve.

Statistical analysis

The obtained results were analysed statistically – arithmetical mean and standard deviation were calculated for measurable traits and for qualitative traits – their quantitative-percentage distribution.

For the measurable traits in conformity with normal distribution, evaluated with Kolomogorov's conformity test, for comparison between the groups unifactor variation analysis was applied and for traits inconsistent with this distribution we applied respectively Kruskal-Wallis' test for many groups. In our calculation $p<0.05$ was assumed statistically significant.

The calculations were made with the statistical software SPSS 8.0 PL and Statistica 6.1 pl. Sensitivity and specificity were calculated and ROC curve was drawn.

Results

In the group of 40 examined children – 30 with diagnosed bronchial asthma and 10 without any symptoms (control) TOP CAST Paediatric Allergen Mix test was performed according to the manufacturer's instructions.

The concentration of leukotrienes (P) released under the influence of the mixture of 21 allergens (inhalatory and food) used in dilution 100 ng/ml and 10 ng/ml was considerably different in the group of children with diagnosed atopic asthma (1) than in the group of patients with non-atopic asthma (2)

Figure 1. **Leukotrienes values (P1) generated under the influence of allergens (concentration 100 ng/ml) in a group of children with atopic asthma (1), non-atopic asthma (2) and control group (3)**

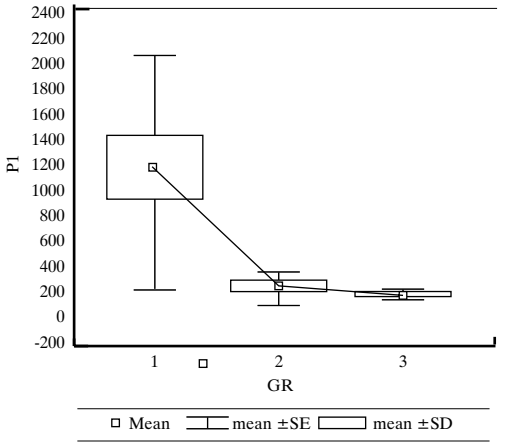


Figure 2. **Leukotrienes values (P1) generated under the influence of allergens (concentration 10 ng/ml) in a group of children with atopic asthma (1), non-atopic asthma (2) and control group (3)**

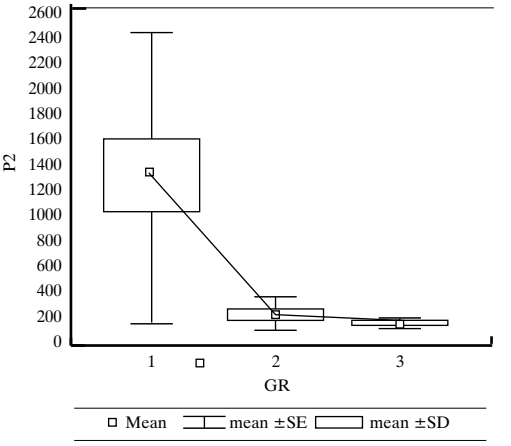


Figure 3. **Sensitivity and specificity of TOP CAST at allergen concentration 100 ng/ml**

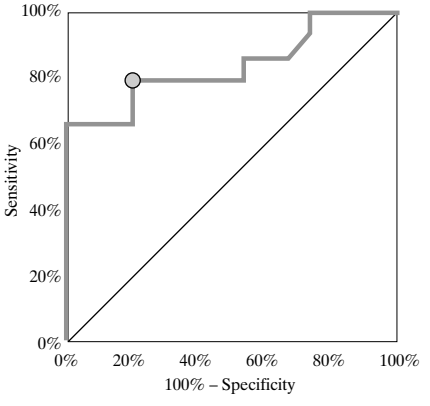


Figure 4. **Sensitivity and specificity of TOP CAST at allergen concentration 10 ng/ml**

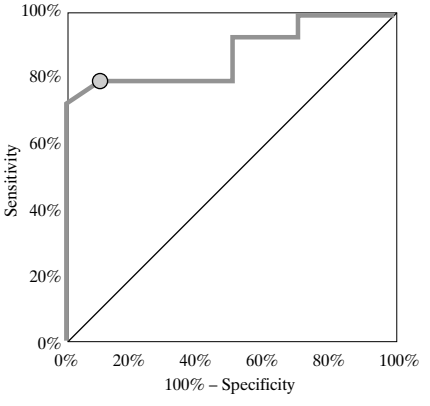
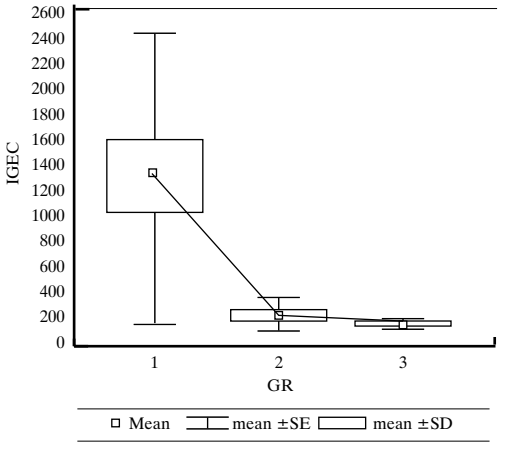


Figure 5. **Total IgE concetration in the group of children with atopic asthma (1), non-atopic asthma (2), and control group (3)**



or control group (3) (*Fig. 1, 2*). Mean concentration of leukotrienes P1 and P2 (at dilution of allergens 100 ng/ml and 10 ng/ml) were respectively (1)-1174 pg/ml (SD=973.8) and 1308 pg/ml (SD=1162), (2)-152 pg/ml (SD=81.5) and 179 pg/ml (SD=83.8), and in control group (3) – 117 pg/ml (SD=24.9) and 144.5 pg/ml (SD=44.8).

There was a statistically significant difference in the value of P1 (0.0014) and P2 (0.0021) between 1/2 and 1/3 group, but no statistically significant difference between 2 and 3 group (non-atopic asthmatics and controls).

Sensitivity and specificity of TOP CAST Paediatric Allergen Mix test for the value of P1 were 80% each, while for P2 – 80% and 90% respectively (*Fig 3*, *4*).

Total IgE concentration was different in group 1 (mean 1123 IU/ml, SD=1462) and 2 (78.8 IU/ml, SD=88.5) and group 3 (15.1 IU/ml, SD=10.6) ($p<0.05$) (*Fig. 5*).

Comparison of skin prick test (wheal size and number of positive results in skin tests) and leukotriens concentration didn't reveal any statistically significant differences ($p=0.831$ and $p=0.774$).

Discussion

For over 30 years the diagnosis of atopic diseases is based on skin tests and evaluation of allergen-specific E class antibodies. Other methods, like tests evaluating basophils reactivity (histamine release test) are not widely used in allergological examinations. In the 1990s, Buhlmann laboratory elaborated a new method of cell tests (CAST ELISA), which is based on measurement of cysteinyle leukotriens generated by bazofiles stimulated by allergens at the presence of IL-3 [1,2].

So far this test has been used in diagnosis of allergic diseases (hypersensitivity to pollens, mites, insect venoms, drugs, polysaccharides) and to monitor immunotherapy in adult patients [3-13]. Our own experience proved applicability of this examination in diagnosis of mite asthma [14] and allergy to tree and grass pollens [15-16] in puerile population.

One application of this method is TOP CAST Paediatric Allergen Mix test, evaluating atopic status of patients. In this test peripheral blood leukocytes are stimulated with a mixture of 21 common inhalatory (grass mix, wheat, birch, hazel, mugwort, ribwort, *Alternaria sp.*, home dust and flour mites, dog, cat) and food allergens (egg, milk, fish, nuts, soy). In literature data regarding applicability of this test is incomplete and only few authors [17-23] have attempted to evaluate usefulness of this method in adult and juvenile patients.

The aim of our study was to evaluate the applicability of this test as screening examination in the direction of atopy in juvenile population with clinical manifestations of bronchial asthma.

For the study, children with diagnosed bronchial asthma were qualified on the basis of history (character of symptoms, recurrence and seasonal incidence), physical examination (typical asthmatic symptoms), functional examination of the lungs (bronchial obturation features, positive reversibility test). All the patients had skin prick tests performed with the most popular aero- and troph-allergens. In the group of children with positive skin tests, allergen-specific IgE was determined in the direction of the allergen triggering disease symptoms. TOP CAST Paediatric Allergen Mix test was performed with two allergen concentrations – 100 ng/ml and 10 ng/ml, according to recommendations of other authors [3,9,10]. In all patients with atopic asthma symptoms [1] we observed wide range of generated leukotrienes both at low and high allergen concentration, mean 1 174 and 1 308 pg/ml respectively and these values were significantly higher compared to the group of children with non-atopic asthma (2): 152 and 159 pg/ml and control group (3) where leukotrienes release under the influence of these allergens at both concentrations was below the test sensitivity (117 and 145.5 pg/ml).

Statistically significant difference of the value of P1 and P2 was found between these groups ($p<0.0014$ and $p<0.0021$ respectively).

Most authors have a positive opinion of CAST ELISA test in diagnosis of allergic ailments in case of single allergens or groups of allergens [1-3,7-8,11-13]. However, very scarce are studies regarding application of this test as a screening examination (mixture of 21 inhalatory and food allergens) in case of suspected atopic diseases, including exogenous asthma, especially in children [17-23].

In our own study sensitivity of TOP CAST Paediatric Allergen Mix test was 80% at both allergen concentrations (100 and 10 ng/ml), but specificity was better at lower concentration – 90%. In available literature the sensitivity of this test was determined at 86-100%, and the specificity at 80-90%, which makes the value of this test comparable to other multitests, such as, for example, Phadiatop [17-23].

We found correlation between total IgE concentration and quantity of released leukotrienes, however, there was no significant difference between the size of the wheal or number of positive results in skin tests and level of leukotrienes. This fact may be explained by qualitative differences of the allergens composition that was used in reagents for skin tests and in TOP CAST test and specificity and sensitivity of both methods.

To sum up, TOP CAST Paediatric Allergen Mix test could be used as a screening examination of the first choice in diagnosis of atopic ailments, especially in small children. This examination could also make a valuable diagnostic tool, e.g in cases when skin tests are impossible to perform (age restrictions, applied treatment, atopic dermatitis) which frequent in the population of developmental age, or where there are discrepancies between the history, skin test results and *in vitro* examinations.

Conclusions

1. TOP CAST Paediatric Allergen Mix test is a good screening method in differentiation of atopic and non-atopic background of bronchial asthma in children.
2. At the current stage of this test evaluation it is used as a complementary method of routine examinations in allergological practice.

Acknowledgements

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Functioning of primary health care in opinion of managers of primary health care units

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Abstract

Purpose: The aim of the research is to get to know opinions of primary health care managers concerning working of primary health care and concerning quality of medical services offered by family doctors out-patient clinics.

Material and methods: The research among managers of primary health care units took place in all out-patient clinics in Lublin province. Research instrument was survey questionnaire of authors own construction. Results were statistically analyzed.

Result: From 460 surveys sent, 108 questionnaires were accepted to analysis.

Majority of managers of out-patient clinics of primary health care is satisfied with the way and the quality of work of employed staff. In opinion of 71.3% of managers access to family doctor services is very good. Availability of primary health care services is better estimated by managers of not public units. The occupied local provide comfortable work for the staff in opinion of 78.5% of surveyed managers of out-patient clinics. Managers estimate the level of their services as very good (37.96%) and good (37.96%) comparing to other such a subjects present in the market. Internal program of improving quality is run in 22% of out-patient clinics, which were investigated.

Conclusions: Managers of primary health care units assess the quality of their services as good and very good. They estimate positively the comfort and politeness in serving patients as well as technical status of equipment and the lodging. They assess availability of their services as very good. Large group of managers of family doctors practices recognizes neighborhood practices as a competitors.

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Key words: primary health care, manager, quality of medical services.

Introduction

The transformation of multispecialistic system of primary health care in Poland into model of family medicine needed special actions. Their starting point was the document "Spectrum of competences of family doctor" accepted by Ministry of Health and Social Care in 1993. This document is an attempt of distinguishing duties of family doctor as a general practitioner and competences of other specialists present in health care system.

Central place in new shaped primary health care should provide efficiency in using public finances intended for health care system. Primary health care units should be independent subjects, fully responsible for both, medical and economical effects of their actions. Independent primary health care units in health care system would be included in public health care system by contract with suitable buyer. Only such a location of family doctor in health care system would be giving a guarantee of rational decisions, which are being taken by family doctor. This would be giving the possibility of improving effectiveness of using finances intended for primary health care system.

Aim of the research

The aim of the research is to get to know opinions of primary health care managers concerning working of primary health care and concerning quality of medical services offered by primary health care units.

Material and methods

The research took place in all outpatient clinics in Lublin province, which signed the agreement with National Health Found in the year 2003 in range of primary health care. Proper

research was followed by pilotage research in 2002. The research was individual and performed with the help of survey questionnaire of authors own construction. The research was anonymous, without the presence of enumerator to avoid false answers. Surveys were sent by regular mail to managers of outpatient clinics of primary health care. Authors kindly asked addresses to fill the survey and resend it to Department of Management and Health Care Economics in Medical University in Lublin (mail stamp and return envelope was attached to every survey).

Results were statistically analyzed with Statistica 6.0 program. Test of homogeneity Chi^2 was used to detect the presence of differences between analyzed groups for nominal features. Test of independence χ^2 was used to find the significance between two variables. The strength of relation was estimated by V-Crammer's factor. Far small numbers we used Yates correction.

Together with pilotage research 460 surveys were sent. Pilotage research was not taken into consideration in statistical analysis.

Among 410 surveys of proper research we have received 134 (29.13% surveys returned). Not fully filled and not properly filled surveys were rejected. 108 questionnaires were accepted to analysis.

Results

Characteristics of investigated group of doctors

108 doctors working as managers of primary health care units were examined.

There were 54.6% (59) women and 45.4% (49) men in investigated group. Majority of persons was age between 41 and 50 (51.9%), 27.4% was age up to 40, minority of persons was age more 51 (20.75%).

Primary health care units, in which investigated physicians are employed, are located in the country (53.7%), in the city (28.7%) and 17.6% in small towns.

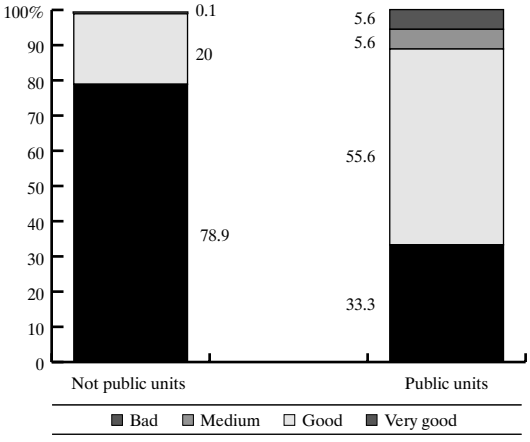
Majority of surveyed doctors was specialized in two fields (59.3%). Large group of doctors had one specialization (29.6%), more than 10% of surveyed was specialized in more then two fields. Taking into consideration length of working in primary health care, majority of surveyed doctors worked as family doctor (or as a GP) more than 15 years (61.1%). Part of investigated managers of primary health care units works in primary health care from 11 to 15 years (17.6%) and from 5 to 10 years (13.9%). Only 7.4% of physicians works less than 5 years.

83.3% of surveyed doctors are managers of not public units, 16.7% are managers of public units.

Doctors, who took part in research, administer primary health care units by their own (73.15%), 26.85% of surveyed managers run their units with partner or are subordinated by the director of the unit.

The majority of investigated primary health care units was employing not more than 10 persons (58.9%). In one third of the units between 11 and 20 persons were employed (33.6%) and more than 20 persons were employed in 7.5% of units.

Figure 1. **Availability of family doctor's service in opinion of managers depending on type of unit**



Functioning of primary health care

Majority of managers of primary health care units is satisfied with the way and the quality of work of employed staff. 47.2% of managers is fully satisfied with the way of the work of the staff as well as with quality of heir work. 46.3% of managers is satisfied but not concerning to all of the persons. 5.6% of managers are satisfied but they see the need of reducing one or more persons. Only one investigated manager is not satisfied with the work of his/her personnel.

Gate keeper role provides equality in access to health services in opinion of 56.5% of surveyed doctors. 32.4% of managers do not have any opinion concerning this matter. 11.1% of surveyed physicians has opinion that it does not make access to medical services easier.

In opinion of 71.3% of managers of primary health care access to family doctor services is very good. About 26% of investigated doctors do think that access is good. Only 1.8% of doctors assess the services of primary health care as an average and only one person assess it badly. These opinions were similar to patient's opinions. Researches performed in Gdańsk and Sopot, patients, who often visited their family doctor estimate it's availability as a very good (50%) and good (42%). Patients who are not often visiting their doctor estimated it's availability as very good in 35% and good (54%) [4].

Researches which took place in England showed that doctors focused on communication to diagnose and plan the treatment of the patients. Doctors did not find the problem of availability in the same way as their patients. Only small group of family doctors discussed this subject with their patients [5].

Availability of primary health care services is better estimated by managers of not public units (78.9% – very good), only 33.3% of managers of public primary health care units estimate availability very good ($p<0.05$), *Fig. 1.*

Half of surveyed managers (51.85%) for the question concerning the need of employing receptionist, who organizes the work in the primary health care units, answered that there is no such a necessity. On fourth of surveyed managers has already employed such a person and find it helpful (18.5%).

Interesting is the fact that there is more managers of not

Figure 2. **The need od employing receptionist in the opinion of surveyed persons depending on type of unit**

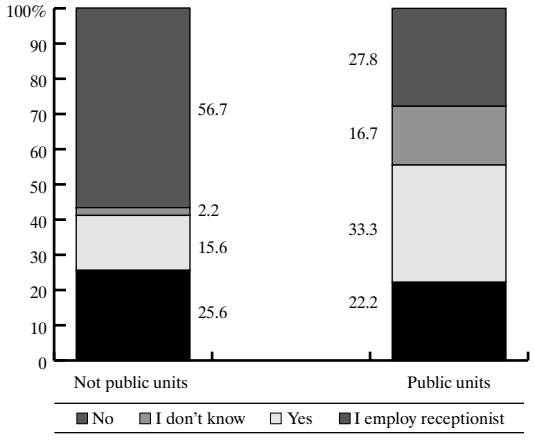
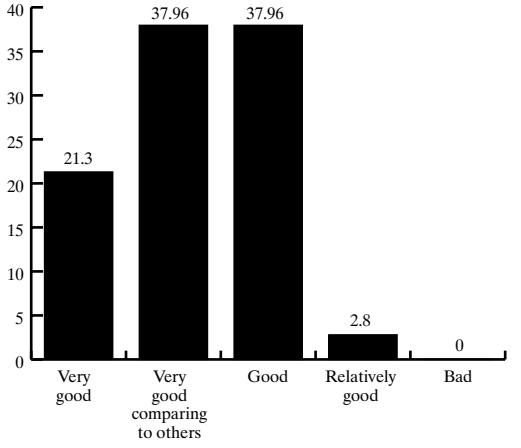


Figure 3. **How high is the level of quality of your unit's services?**



public units (56.6%) who do not find the need of employing the person responsible for organizing work than managers of public units (27.8%) ($p<0.05$), *Fig. 2*.

Majority of investigated doctors have opinion that family doctors are well and comprehensive prepared to their function (77.8%). 22.2% of examined doctors do think that family doctors are not enough prepared to their work.

The occupied local provide comfortable work for the staff in opinion of 78.5% of surveyed managers of primary health care units. One fifth of examined (19.6%) do think that the local does not cover the expectations of workers.

Majority (74.8%) of managers has also opinion that the lodging provides comfort for working with patient. About 14% do think that the comfort is not full. 11.2% of managers do not have any opinion concerning this, because there was no research among patients in this matter. The technical status of the rooms was estimated by patients as good (64%) and bad (18%).

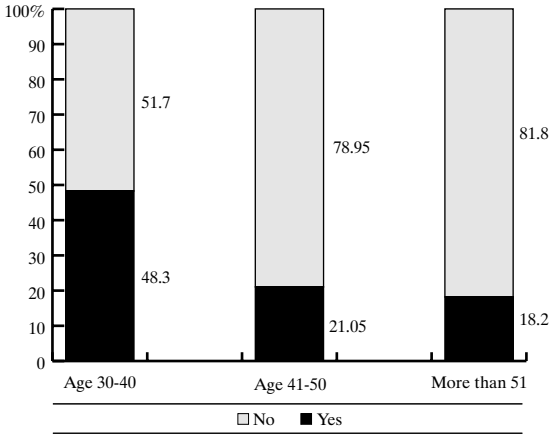
Although fulfilled all of expert and sanitary requirements concerning lodging, 41.1% of managers mentioned that driveway for disabled persons are still not present. Most different situation in this aspect is in the country. 5.6% of primary health care units are not available for persons on wheelchairs. The problem touch 25.8% of primary health care units in the cities and 31.6% in small towns.

Managers of primary health care units estimate the level of their services as very good (37.96%) and good (37.96%) comparing to other such a subjects present in the market which is comparable with patients opinion, *Fig. 3*.

In the researches of other authors performed among patients of two primary health care units: public one and not public one, respondents estimated two times often the health care in not public units as better to compare with public units [7]. In the opinion of patients better care was associated with better communicativeness of doctors and better management of time of the work.

Managers of primary health care units do think that employees do not have motivation to perform their services in higher level, because of their law salaries and law comfort of their work (54.6%). 45.4% has opposite opinion about it. More than

Figure 4. **Are you as a manager well paid? (depending on the age of surveyed persons)**



70% of managers has opinion that they are not well paid. Dissatisfaction with the salary grows with the age of the manager ($p<0.05$). The most dissatisfied are doctors in alder age group (81.8%) and the least dissatisfied are doctors in age group up to 40 (51.7%), *Fig. 4*.

Opinions concerning the salary of family doctors in Podlasie area were different depending on the kind of unit of health care.

Doctors working in not public units (contracted) are in 62% satisfied with their salaries and only 8% of public units doctors have the same opinion.

More or less 57% of surveyed doctors say that peer group meetings are organized to improve the level of quality of services in primary health care and personnel is involved in these meetings. 27.1% of managers said that such a meetings do not take place. 15.9% have no piece of information about it. The least interest in meetings are persons older than 51 years (45% answers – yes, 45% answers – no). Most involved in improving the quality of services by exchanging experiences are managers in the age between 41 and 50 years old (66.1%, $p<0.05$), *Fig. 5*.

Figure 5. **Are peer group meetings organized? (answers depending on the age of surveyed persons)**

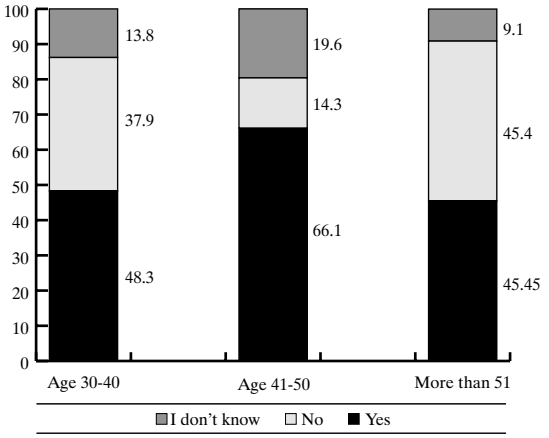
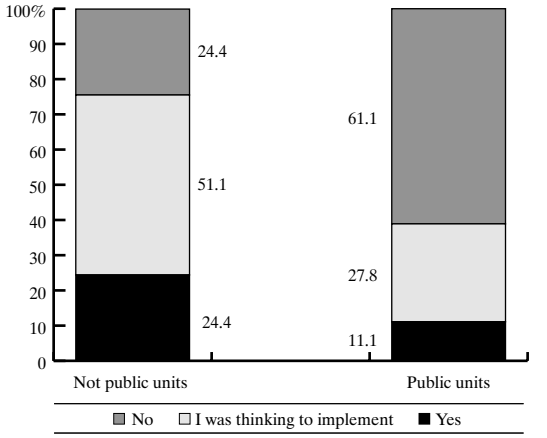


Figure 6. **Are you running an internal program of improving quality? (answers depending on the type of unit)**



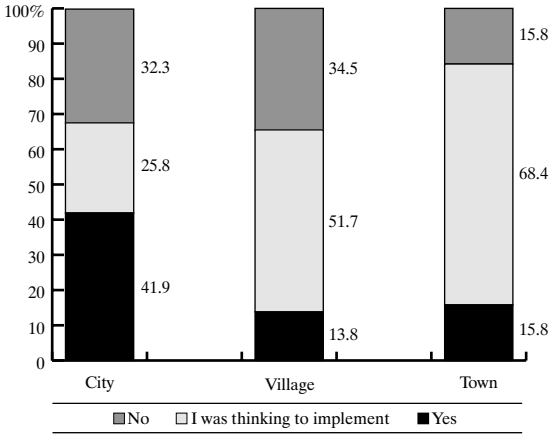
Internal program of improving quality is run in 22% of primary health care units, which were investigated. Plans of implementing such a program have about 47.2% of managers who are responsible for quality of services in their units. 30.6% of managers of family doctors practices do not run any internal program of improving quality of services and do not plan to implement such a program.

Bigger interest in improving the quality of services we can observe in not public primary health care units. 24.4% of managers have already running internal program of improving the quality of services and 51.1% of theme is planning to implement such a program. Among public units such a program is run in 11.1% and is being planned in 27.8% of units ($p<0.05$). The highest percentage of managers, who implemented the internal program of quality of services is in units located in the city (41.9%, $p<0.05$), *Fig. 6*.

90.74% of managers mention that actions improving health are being organized, 9.26% deny of the presence of such actions. Answers of surveyed managers concerning this matter depended on kind of unit they managed. Almost all (94.45) of managers of not public units answered that health improving actions for patient are organized in their units. 27.8% of managers of public units did not organized such a actions ($p<0.05$). Among questioned patients only 25% confirmed their parting in such actions, *Fig. 7*.

For question about innovative ideas with improving health direction and differentiate given unit from the others 45.37% of surveyed managers answered that they do not have enough founds for such an activity. 40.74% mentioned that they do not have these kind of ideas. 13.89% see possibility of such action and is in progress with preparations. 75.93% of questioned managers did not examined real needs of their patients. 24.07% of managers have already checked needs of their patients.

Figure 7. **Are you running an internal program of improving quality? (answers depending on place of location of the unit)**



Conclusions

1. Managers of primary health care units assess the quality of their services as good and very good. They estimate positively the comfort and politeness in serving patients as well as technical status of equipment and the lodging. Managers of primary health care assess availability of their services as very good. Following manager's opinions patient can easy receive appointment in suitable time for theme.

2. Around half of the managers of primary health care do not see any need of rationalizing their units with employing receptionist. In a small degree there are implemented internal programs of improving the level of quality in out-patient clinics.

3. Examining the satisfaction of patient is not important element in running of primary health care units, because only a few units lead investigations concerning needs of their patients.

4. Large group of managers of primary health care units recognizes neighborhood practices as a competitors, which motivates theme to implement any innovative projects in their units.

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Alternative therapies in antibiotic-resistant infection

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Abstract

Case report: A 24-year-old woman suffering from post-influenza otitis media infection was initially treated with several series of a steroid (Elocon) and a combination of steroids and antibiotics (Atecortin, Dicortineff) without significant medical benefit. The isolated bacterial strains were identified as *Staphylococcus homis* and *Staphylococcus epidermidis*. Specific phage therapy applied sequentially over a period of three weeks resulted only in a partial reduction in inflammation and limited improvement in overall health condition. Oral application of lactoferrin (LF; 50-mg daily oral doses for seven days with two-week intervals) led to a complete clearance of both bacterial strains and full recovery of the patient. The recovery was associated with increased myelopoiesis and a sustained elevation of serum endogenous LF. In conclusion, specific bacteriophage therapy combined with the administration of lactoferrin proved to be effective in the treatment of antibiotic-resistant external ear infection.

Key words: infection, inflammation, antibiotic resistance, bacteriophages, lactoferrin.

Introduction

The renaissance of interest in phagotherapy in recent years is associated with the aggravating problem of resistance of bacterial strains to antibiotics [1]. Phage therapy is highly effective in curing suppurative infections otherwise resistant to antibiotic therapy and provides long-term specific resistance (for review, see [2]). Recently we also demonstrated that lactoferrin (LF), an iron-binding protein contained in the neutrophils and secretory fluids of mammals, provides adjunct therapy benefits in various experimental designs in mice [3-5]. Our earlier investigations on human volunteers [6] revealed that LF, given orally, regulates several immune parameters in healthy subjects, including a rise in the percentage of circulating neutrophil precursors. LF has also been shown to exhibit direct and indirect antibacterial actions [7]. The aim of this report was to test the effectiveness of combined bacteriophage and LF therapy in a patient with external ear infection.

Case

The investigation was conducted on a 24-year-old woman suffering from post-influenza otitis media infection. The patient was initially treated topically with a steroid (Elocon) and a combination of steroids and antibiotics (Atecortin, Dicortineff). None of the applied drugs proved effective. The patient then reported (April 12, 2000) to the Laboratory of Bacteriophages for the isolation and identification of the pathogen(s) involved in the infection. The isolated bacterial strains were identified as *Staphylococcus homis* (left ear) and *Staphylococcus epidermidis* (right ear). The patient was subjected to two series (beginning May 12 and June 16, 2000) of oral phage therapy with a phage specific to *Staphylococcus aureus* (676/T) from the collection of the Institute of Immunology [8]. After treatment, the two original bacterial strains were still identified at the sites of inflammation. Therefore, the patient was additionally treated (July 2000) with bacteriophages specific to *Staphylococcus* (A3/R and 676/T) for three weeks, both orally and topically in the form of droplets. Although some improvement in health condition was registered by endoscopic examination, the patient complained of persisting pain and discomfort. Eventually the patient was subjected to oral treatment with bovine lactoferrin in capsule

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Table 1. **The studied clinical parameters during therapy in the patient**

Parameters	Phage therapy		LF treatment	
	before treatment	after 4 months of treatment	one week after treatment	two months after treatment
leukocytosis (mm^3)	4600	5000	4800	5100
phagocytic index (%)	72	71	57	73
serum LF (ng/ml)	168	238	930	753
neutrophil precursors (bands) (%)	3	4	7	10
neutrophils (%)	54	54	52	48
basophils (%)	2	1	1	2
eosinophils (%)	3	1	2	1
monocytes (%)	0	0	0	0
lymphocytes (%)	38	40	38	39

form (50 mg daily for seven days, three repeats with two-week intervals). The lactoferrin was a gift from Pharma Review, Houston, USA. The undesired symptoms alleviated in the course of the LF treatment.

Results

Tab. 1 illustrates the alterations in the magnitudes of the studied parameters during the first series of phage therapy and during treatment with LF. The results show that the levels of neutrophils and their precursors as well as the level of serum lactoferrin and the neutrophils' ability to phagocytize *Staphylococcus aureus* did not significantly change after three weeks of phage therapy. However, following LF treatment the values of the studied parameters began to increase (*Tab. 1*). Some of the changes were even more profound two months after treatment and included a very strong increase in the level of neutrophil precursors, to 10% of the total cell count, accompanied by a decrease in mature neutrophils and still a significant elevation in serum LF concentration (an over threefold increase).

Discussion

Resistance to antimicrobial agents continues to be a major cause of increased morbidity, mortality, and healthcare costs. The foremost contributing factor to the problem of antibiotic-resistant infections is the widespread use of certain antibiotics. Here we demonstrate the utility of specific bacteriophage and lactoferrin therapy to combat such antibiotic-resistant infections. This study shows a direct interdependence between the effectiveness of antibacterial therapy and changes in the level of endogenous serum LF as well as those of the most efficient phagocytes: circulating neutrophils and their precursors. Although it is difficult to ascertain the exact mechanism of action of LF, in part because other therapeutic agents were used in this protocol, the hitherto accumulated knowledge on LF suggests LF-induced upregulation of proinflammatory mediators. Among these mediators is IL-8, a cytokine responsible for the degranulation of neutrophils [9]. According to the hypothesis of Rich et al. [9], endogenous LF, released from neutrophils, generates a "demand" signal for an increased recruitment of neutrophils from the bone marrow, generated by LF-inducible proinflammatory cytokines including G-CSF (granulocyte-colony stimulating factor). Such a hypothesis may explain the significant increase in lactoferrin serum level and neutrophil precursors in the course of LF treatment (Tab. 1) and the decreased percentage of mature neutrophils. Thus LF accelerates neutrophil turnover. A similar phenomenon was observed in human volunteers treated with LF [6] as well as in an animal model using mice [5]. It is, however, more difficult to explain why the ability of neutrophils to phagocytize bacteria transiently decreased during the treatment with LF. A similar phenomenon was observed in our larger study of patients subjected to phage therapy [10], which could be simply connected with patient recovery and a decreased necessity to phagocytize bacteria. The decreased ability to phagocytize bacteria may also be compensated by the increased recruitment of new neutrophils. Nevertheless, by the end of the treatment the ability of neutrophils to phagocytize bacteria had returned to the levels observed at the beginning of the monitoring (73% vs 72%). The causes of LF therapeutic action in the local infection may be more complex and involve the previously described direct and indirect antibacterial properties of lactoferrin [4-7]. We are of the opinion that the direct antibacterial action of LF is accomplished by the distinctly elevated endogenous (human) LF (*Tab. 1*), as determined by the immunoenzymatic method using antibodies specific to human LF. It is also possible that LF, given orally, may reach distant sites of infection, as was demonstrated in the case of mice with *Staphylococcus aureus* urinary infection [11].

In conclusion, phage therapy, combined with oral lactoferrin treatment, appeared to be effective in curing external ear infection otherwise resistant to antibiotic therapy. The therapeutic effect correlated with a stimulation of myelopoiesis and a rise in serum LF level, parameters crucial in the defense against bacterial infections. However, in the described case, a self recovery should also be taken into account.

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Ultrastructural study of the submandibular gland of the rat after 6-month exposure to cadmium and zinc in drinking water

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Abstract

Purpose: Cadmium toxicity in the exposure of the general, professional and cigarette smoking populations has been well known. From the dental point of view, it is important to find out whether and how separate and joint exposures to cadmium and zinc affect the structure and function of the submandibular gland, which is the major saliva-releasing gland. Cadmium, a particularly active xenobiotic, damages cellular metabolism at the level of various enzymatic systems of the cell, which may disturb functioning of the salivary glands. Mutual interactions of cadmium and zinc suggest a protective role of zinc through the induction of metallothionein which inactivates cadmium effect.

Material and methods: The aim of the study was to assess the ultrastructural picture of chosen cell organelles of the submandibular salivary gland of the rat exposed to cadmium and zinc. The study used 90 male Wistar rats, of the initial b.w. 150-180 g. The animals were exposed to cadmium and/or zinc for 6 months. Cadmium was received in aqueous solutions of cadmium chloride with drinking water at a concentration of 5 mg Cd/dm^3 or 50 mg Cd/dm^3. Zinc was also given in aqueous solutions of zinc chloride ad libitum at concentrations of 30 mg Zn/dm^3 and 60 mg Zn/dm^3.

Results: The ultrastructural changes in the mucous and serous cells of the submandibular salivary gland were most pronounced at cadmium concentration of 50 mg Cd/dm^3 and were mainly observed in the cell nucleus, Golgi Apparatus and secretory granules of the salivary gland cells.

Conclusions:

1. Exposure to cadmium induces ultrastructural changes in the submandibular gland, which are dose and time of exposure-dependent.
2. Exposure to zinc did not affect significantly the ultrastructural picture of cells of the submandibular gland.
3. Zinc administered together with cadmium reduces the intensity of ultrastructural changes in the submandibular gland.

Key words: ultrastructure, submandibular gland, cadmium, zinc.

Introduction

The civilization advances and growing environmental pollution bring the effect of various xenobiotics, including heavy metals, on the functioning of the living organism [1-3]. Cadmium toxicity in the exposure of the general, professional and cigarette smoking populations has been well known [4]. Its effect on long bones, kidneys and liver has been investigated in experimental rat models [5-10]. From the dental point of view, it is important to determine whether and how separate and joint exposures to cadmium and zinc affect the structure and function of the submandibular gland, the major saliva-releasing gland. The oral cavity can be the first site through which the xenobiotic gets via the respiratory and alimentary tract to the body, and the saliva is a "protective coat" for the oral structures. Cadmium, a particularly active xenobiotic, interferes with cellular metabolism at the level of various enzymatic systems of the cell and may thus disturb functioning of the salivary gland. Biological consequences of the effects of the respective metals and their mutual interactions on the structure of submandibular gland cells can be assessed only using animal experimental models. Mutual interactions of cadmium and zinc suggest a protective role of zinc through the induction of metallothionein which inactivates the cadmium effect [7,11].

The aim of the study was to assess the ultrastructural picture of chosen cell organelles of the submandibular gland of the rat exposed to cadmium and zinc. There is no data on an interaction influence of cadmium and zinc together administrated in a chronic poisoning of cadmium.

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Material and methods

The study used 90 male Wistar rats (initial b.w. 150-180 g). The animals were exposed to cadmium and/or zinc for 6 months. Rats received cadmium in the form of aqueous solutions of cadmium chloride with drinking water at a concentration of 5 mg Cd/dm^3 or 50 mg Cd/dm^3. Zinc was also given in aqueous solutions of zinc chloride ad libitum at concentrations of 30 mg Zn/dm^3 and 60 mg Zn/dm^3. Control animals received water to drink without cadmium and zinc. The rats had unlimited access to LSM diet. Throughout the experiment the intake of drinking water and body mass increase were monitored. The rats were divided into 9 groups according to metal concentration: group I – control, group II 30 mg Zn/dm^3, group III – 60 mg Zn/dm^3, group IV – 5 mg Cd/dm^3, group V – 5 mg Cd/dm^3 + 30 mg Zn/dm^3, group VI – 5 mg Cd/dm^3 + 60 mg Zn/dm^3, group VII – 50 mg Cd/dm^3, group VIII – 50 mg Cd/dm^3 + 30 mg Zn/dm^3 and group IX – 50 mg Cd/dm^3 + 60 mg Zn/dm^3. After the exposure termination, the animals were anaesthetised (with Vetbutal) and then, immediately, 4 sections of 1-2 mm^3 vol. each were cut off from the submandibular gland in 3 animals from each group. The sections were obtained from the same part of the organ examined.

The tissue material was collected and prepared for ultrastructural analysis following generally accepted principles. Sections for ultrastructural examinations were fixed in 3.6% glutaraldehyde at a temp. of 4°C for 2 hours, postfixed in 2% osmium tetroxide, dehydrated in alcoholic series, propylene oxide and embedded in Epon 812. Semithin preparations were stained with toluidine blue, while ultrathin sections were contrasted with uranyl acetate and lead citrate, and evaluated in a transmission electron microscope OPTON 900 PC [12]. The experiment was approved by the Bioethical Committee (2004/03).

Results

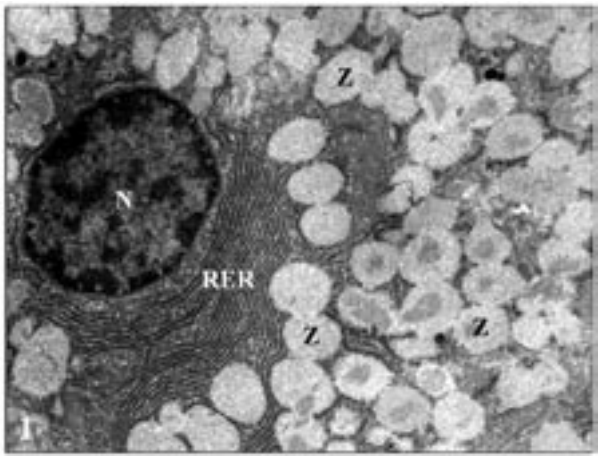
Group I (control)

The ultrastructure of the submandibular gland was normal. Mucous acinar cells had oval nuclei located in the parabasal part. In the vicinity of the nucleus, there were parallel channels of the rough endoplasmic reticulum, Golgi Apparatus, as well as oval or elongated mitochondria with numerous crests and matrix of moderate electron density. In the central and apical part of the cell, bright mucous granules containing microgranular material were seen in great numbers (*Fig. 1*). Cellular membranes of the adjacent cells were indented and linked together. Moreover, the cells were joined together by desmosal junctions.

Serous acinar cells had round or oval nuclei, which were located in the central or parabasal parts. The rough endoplasmic reticulum channels were shorter and less numerous than in mucous cells. In the vicinity of cell nuclei, Golgi Apparatus and many slightly elongated mitochondria could be observed. A large part of the cell was filled with relatively numerous secretory granules of high electron density.

In the stroma, a large number of tiny blood vessels were seen between the vesicles.

Figure 1. **Group I (control). Fragment of normal mucous cells of the submandibular salivary gland. N-cell nucleus, RER – rough endoplasmic reticulum, Z – secretory granules. Mag. x 4400**



Group II (30 mg Zn/dm^3)

The ultrastructural picture of the cells of the submandibular gland in this group did not differ from the control one.

Group III (60 mg Zn/dm^3)

The ultrastructural picture of the cells of the submandibular salivary gland in this group also did not differ significantly from the control one. However, the picture showed numerous secretory granules, both in mucous and serous acinar cells, which accumulated around the smoothed-surface lumen. Mitochondria with slightly increased matrix translucence were seen.

Group IV (5 mg Cd/dm^3)

In the ultrastructural picture of the submandibular salivary gland of rats exposed to low doses of cadmium, cell nuclei had a plicate nuclear membrane with large distinct nucleoli. The Golgi Apparatus was activated and with dilated cisterns. Mitochondria were slightly translucent and only sporadically myelinic structures could be seen within them.

Group V (5 mg Cd/dm^3 + 30 mg Zn/dm^3)

The ultrastructural picture of the acinar cells showed very subtle changes and thus it was not significantly different from the control one. Only in mucous cells, large secretory granules were seen.

Group VI (5 mg Cd/dm^3 + 60 mg Zn/dm^3)

In mucous cells, the nuclei frequently had irregular contours. Serous cells contained plenty of large secretory granules. Condensations of mucous cell granules were rare.

Group VII (50 mg Cd/dm^3)

The nuclei of mucous cells of animals exposed to this high concentration of cadmium had distinctly irregular contours and very large nucleoli (*Fig. 2*). The rough endoplasmic reticulum showed local degranulation and had an irregular course. Golgi

Figure 2. **Group VII (50 mg Cd/dm³). Fragment of mucous cells with numerous secretory granules accumulated around the acinar lumen /L/ with smoothed surface. The nuclear cell /N/ shows plicate margins and a large nucleolus /n/. M – mitochondria exhibited damaged crests. Mag. x 3000**

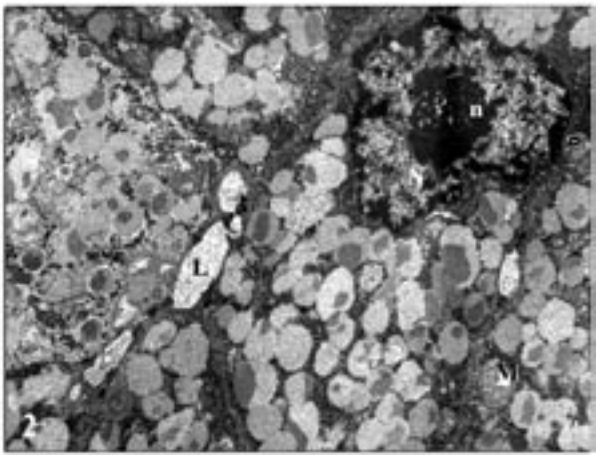
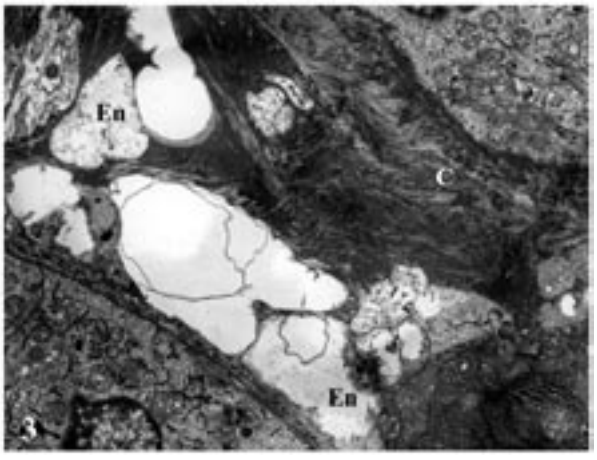


Figure 3. **Group VII (50 mg Cd/dm³). Fragment of blood vessel with damaged endothelial cells /En/. In the vicinity, a number of collagen fibres /C/. Mag. x 3000**



Apparatus was evidently well developed and had dilated cisterns. Numerous mitochondria exhibited damaged crests and matrix of increased translucence. Secretory granules in mucous cells contained numerous irregularly arranged "paracrystalline" condensations, some of which merged. The acinar lumen had a smoothed surface (*Fig. 2*).

Serous acinar cells contained a large number of secretory granules that varied in size. In tissue stroma, blood vessels with badly swollen endothelial cells and an increased number of collagen fibres were seen (*Fig. 3*).

Group VIII (50 mg Cd/dm³ + 30 mg Zn/dm³)

In this group, the nuclei of mucous acinar cells had irregular contours, large nuclei and chromatin condensed on the periphery. Some of the mitochondria showed increased matrix translucence, but with no distinct features of damage. Secretory granules of these cells merged to form "little lakes" of mucosa (*Fig. 4*).

In serous acinar cells, the granules were large and numerous. Increased cytoplasm translucence was sometimes observed. In tissue stroma, blood vessels with swollen endothelial cells and numerous collagen fibres were seen.

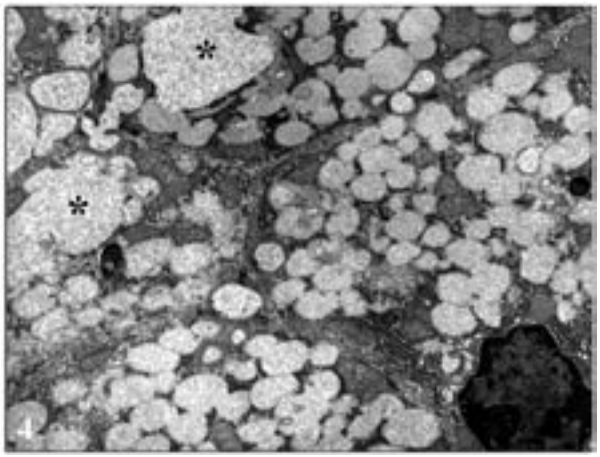
Group IX (50 mg Cd/dm³ + 60 mg Zn/dm³)

The ultrastructural picture of acinar cells was similar to the one observed in group VIII. There were, however, numerous regularly arranged condensations of mucous granules.

Discussion

Cadmium, lead, iron and mercury, known as particularly active xenobiotics, are the focus of many research studies. They manifest toxicity by damaging cell metabolism and disturbing membranous transport, cellular respiration, lipid metabolism and protein synthesis [7,13].

Figure 4. **Group VIII (50 mg Cd/dm³ + 30 mg Zn/dm³). Fragment of mucous cells with secretory granules merging to form the so called "little lakes" /*/. Mag. x 3000**



In the current study, the ultrastructural changes in the secretory cells of the submandibular salivary gland of rats were cadmium concentration-dependent. In the group with lower cadmium intoxication (5 mg Cd/dm³) no significant deviations were found as compared to control. In the rats receiving higher cadmium concentration (50 mg Cd/dm³), the ultrastructural picture showed very distinct damage to cell organelles, with the cell nucleus, mitochondria, Golgi Apparatus and rough endoplasmic reticulum being affected. The nuclear membrane was plicate and the number and size of nucleoli were increased, which may indicate activation and intensification of metabolic functions of the salivary gland cells. Degranulation of the rough endoplasmic reticulum and various degrees of mitochondrial damage could be seen, usually with swelling and increased translucence of the matrix. Czykier et al. [14], studying the submandibular

salivary gland in acute cadmium intoxication (50 mg Cd/dm^3) also observed nuclear membrane plication and Golgi Apparatus activation. However, at a concentration of 100 mg Cd/dm^3 these authors described secretory granules merging to form "little lakes" and with no damage to organelles, which may suggest the development of adaptive mechanisms or increased saliva secretion. Also in the current study, we observed an increase in the quantity of secretory granules varying in shape and accumulating around the acinar lumen. Smoothing of the acinar lumen and accumulation of numerous secretory granules may indicate a cadmium-induced disturbance in calcium ion homeostasis and membranous transport [15-17]. Damage to mitochondria can cause disturbances in cellular respiration, and degranulation of the rough reticulum may suggest disorders in the synthesis of the cell-specific proteins. Cadmium, competing with other metals in metalloenzymes affects protein synthesis, energetic processes, membranous transport, metabolism of lipids and nucleinic acids [17]. This metal reacting with the SH groups of proteins inactivates cytosolic and mitochondrial enzymes, thus reducing the activity of main mitochondrial enzymes – succinic dehydrogenase and cytochrome oxidase, which interferes with the processes of oxidative phosphorylation [18]. Changes in the biochemical processes of the Krebs cycle, due to cadmium binding to SH-groups, lead to a decrease in ATP and to metabolic deficiency of the cell. This may occur even at a low cadmium exposure [15]. In the cell, cadmium binds to proteins of the cytoplasm, mitochondrial and lysosomal membranes, and cell nuclei, which may affect the membranous transport of the cell [18]. Literature evidence seems to suggest that the toxic effect of cadmium can be accompanied by the formation of reactive oxygen forms which may exert a negative effect on cell metabolism [7].

Morphological changes of various degrees have been also observed in the salivary gland cells exposed to lead and were dose and exposure time-dependent [19]. In the cells of the parotid salivary gland of the rat, the authors described plication of the nuclear membrane, chromatin clumping, dilation of RER channels, Golgi Apparatus activation and features of considerable mitochondrial damage. They also found large lipid deposits and a smaller numbers of secretory granules in comparison to control.

Morphological changes have been noted in the liver and kidney due to cadmium exposure [20]. Among the early lesions in the kidney, swollen mitochondria with condensed matrix predominated and proliferation of smooth endoplasmic reticulum could be seen [8]. The ultrastructural picture of hepatocytes chronically exposed to cadmium showed an increase in perichromatin granules in the nuclei and nucleolar condensation [9]. This may suggest various mechanisms of metal actions and different cell "sensitivity" to a xenobiotic [21].

The combined administration of cadmium and zinc in the current study showed a protective role of zinc during exposure to cadmium. In the ultrastructural examination of the submandibular salivary gland exposed to cadmium + zinc, the latter reduced the intensity of morphological changes in the cells. With an increase in zinc concentration and no change in the level of cadmium, lesions in cell organelles were decreased. Numerous condensations in regularly arranged mucous granules indicated normalization of secretory functions of the submandibular salivary gland.

According to some authors, normalization in the picture of the salivary gland exposed to the simultaneous action of both metals may result from different mechanisms of the effect of zinc or can be due to interactions between the metals [22]. The interactions between zinc and cadmium can be explained by their affinity to metalloenzymes. Zinc is involved in many metabolic processes in the body and is necessary for their normal course. Adequate zinc concentration conditions the activity of over 200 enzymes. Due to its presence in many enzymes, zinc controls the pro- and antioxidative balance [23,24]. At high concentrations, zinc can also show antioxidative effects, regardless of the enzymes it builds. In the extracellular spaces, zinc protects sulphydryl groups against oxidation and prevents generation of reactive oxygen forms in the presence of such transitory metals as cadmium [25].

Conclusions

1. Exposure to cadmium induces ultrastructural changes in the submandibular gland, which are dose and time of exposure-dependent.
2. Exposure to zinc did not affect significantly the ultrastructural picture of the submandibular gland cells.
3. Zinc administered together with cadmium reduces the intensity of ultrastructural changes in the submandibular gland.

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Ureaplasma urealyticum and *Mycoplasma hominis* infection in women with urogenital diseases

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Abstract

Purpose: The aim of the study was to assess the incidence of *Ureaplasma urealyticum* (*U. urealyticum*) and *Mycoplasma hominis* (*M. hominis*) infection in women with urogenital diseases.

Material and methods: *M. hominis* and *U. urealyticum* was assessed in 541 women from gynaecological and STD outpatient clinics, aged 18-55 years. A Mycoplasma IST 2 kit was used for biochemical determination of mycoplasmal infections (BioMerieux). Additionally, 248 of patients were examined for *Chlamydia trachomatis* (*C. trachomatis*), *Trichomonas vaginalis* (*T. vaginalis*) and *Candida albicans* (*C. albicans*) infection. *C. trachomatis* was detected by direct immunofluorescence method. The standard culture methods (Biomed) were applied to detect *T. vaginalis* and *C. albicans*.

Results: *U. urealyticum* was detected in 161 (29.8%), and *M. hominis* in 20 (3.7%) women. *U. urealyticum* infection alone was observed in 37/79 (46.8%), and 1/8 (12.5%) patient had only *M. hominis* infection. The *U. urealyticum* infection showed most frequent coexistence with *C. albicans* (29.1%), and less frequent with *C. trachomatis* (13.9%) and *M. hominis* infection (10.1%). The highest percentage of mycoplasma-positive cultures was found in patients of STD clinic and in infertile women. In patients with ureaplasmal infection only the most common clinical symptom was vaginal discharge and vulval/vaginal irritation. In 8.1% of the women, the course of *U. urealyticum* infection was asymptomatic.

Conclusions: The incidence rate of genitourinary infections due to *U. urealyticum* was significantly higher as compared to *M. hominis* infection. Sexual mycoplasmal infections were most frequently reported in the group of patients of STD clinic and correlated with age and sexual activity.

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Introduction

The aim of the study was to assess the incidence of *Mycoplasma hominis* (*M. hominis*) and *Ureaplasma urealyticum* (*U. urealyticum*) in women with clinical symptoms of inflammation conditions of the genitourinary organ.

Mycoplasms constitute a large group of microorganisms, but only some, i.e. Mycoplasma and Ureaplasma species, are pathogenic for humans. So far detected in humans, they mainly inhabit the mucous membranes of the respiratory tract and genitourinary system. Three species have been isolated from the mucosal surfaces of the genitourinary tract: *M. hominis*, *U. urealyticum* and recently discovered *Mycoplasma genitalium* (*M. genitalium*) [1]. They are commonly referred to as "genital mycoplasms", as the infection occurs via sexual contacts.

The role of mycoplasms in aetiopathogenesis of inflammatory states of the genitourinary organ is still a subject of controversy. Their presence has been associated with the incidence of urethritis, vaginitis, cervicitis, pelvic inflammatory disease (PID) and pathology of pregnancy and newborns [2]. Mycoplasms are also known to be part of the comensal flora of the genitourinary tract mucosa and are found in the majority of sexually active humans [3,4].

Material and methods

The study group consisted of 541 women aged 18-55 years (mean 29.5 years), with clinical symptoms or suspected of genitourinary tract infection, who were referred to the Diagnostic-Research Centre of Sexually Transmitted Diseases (STD) from gynaecological and STD outpatient clinics in North-eastern Poland for microbiological diagnostics. The patients reported with the following diagnoses: vaginitis – 148, cervicitis with or

***Table 1*. Detection of *M. hominis* and *U. urealyticum* in particular groups of studied women (n=541)**

Studied group	*M. hominis*		*U. urealyticum*	
	+ n (%)	- n (%)	+ n (%)	- n (%)
patients of STD clinic (n=22)	2 (9.1)	20 (90.9)	13 (59.1)	9 (40.9)
infertility (n=43)	1 (2.3)	42 (94.7)	16 (37.2)	27 (62.8)
colpitis (n=148)	6 (4.1)	142 (95.9)	53 (35.8)	95 (64.2)
cervicitis with erosion or without (n=80)	3 (3.8)	77 (96.2)	23 (28.7)	57 (71.3)
unsuccessful pregnancy (n=70)	2 (2.9)	68 (97.1)	19 (27.1)	51 (72.9)
pelvic inflammatory disease (n=75)	4 (5.3)	71 (94.7)	18 (24.0)	57 (76.0)
urethral syndrom (n=103)	2 (1.9)	101 (98.1)	19 (18.4)	84 (81.6)

***Table 2*. Coinfection of *U. urealyticum* with others sexually transmitted pathogens (n=79)**

Pathogen	Coinfection n (%)
U. urealyticum + *C. albicans*	23 (29.1)
U. urealyticum + *C. trachomatis*	11 (13.9)
U. urealyticum + *M. hominis*	8 (10.1)
U. urealyticum	37 (46.8)

***Table 3*. Clinical symptoms in women testing positive for *U. urealyticum* only (n=37)**

Symptom	n (%)
vaginal discharge	16 (43.3)
vulval or vaginal irritation	7 (18.9)
lower abdominal pain	4 (10.8)
dysuria	4 (10.8)
contact bleeding	2 (5.4)
dyspareunia	1 (2.7)
asymptomatic course	3 (8.1)

without erosion – 80, urethral syndrome – 103, pelvic inflammatory disease (PID) – 75, gestational pathology (miscarriage, preterm labour) – 70, and fertility impairment – 43. In 22 women, tests were done for epidemiological reasons (casual sexual relationship, patients of STD clinic).

Material for analysis included smears taken from the uterine cervix and/or urethra, and from vaginal fornix posterior. A Mycoplasma IST 2 kit was used for biochemical determination of mycoplasmal infections (BioMerieux). This test allows identification of mycoplasmal pathogens within 48 h and estimation of the amount of bacteria to differentiate between colonization and infection ($>10^4$ cells – evidence of infection). It also makes it possible to evaluate antibiotic sensitivity of the microorganisms. In 248 women, a direct immunofluorescence method with monoclonal sera (Trinity Biotech) was used for the diagnosis of *Chlamydia trachomatis* (*C. trachomatis*) infection and standard culture methods (Biomed) were applied to detect *Trichomonas vaginalis* (*T. vaginalis*) and *Candida Albicans* (*C. albicans*). Bacterial vaginosis (BV) was diagnosed according to Amsel's criteria and after macroscopic evaluation of the Gram-stained smears. *Neisseria gonorrhoeae* infection was detected through a microscopic evaluation of Gram-stained smears. The study was approved by the Bioethics Committee, Medical University of Białystok.

Results

In the study group of 541 women, *U. urealyticum* was detected in 161 (29.8%), while *M. hominis* in 20 (3.7%) cases. In all the patients, *Neisseria gonorrhoeae* infection and bacterial vaginosis were excluded. The incidence rates of sexual mycoplasms in the respective groups have been presented in *Tab. 1*. The highest percentage of mycoplasm-positive cultures was found in patients of STD clinic. *U. urealyticum* was detected in 13/22 (59.1%), while *M. hominis* in 2/22 (9.1%) patients. The second highest incidence was observed in patients with fertility impairment. *U. urealyticum* was found in 16/43 women (37.2%) and *M. hominis* in 1/43 (2.3%) patient. The third were the patients with colpitis symptoms. *U. urealyticum* was isolated in 53/148 (35.8%) and *M. hominis* in 6/148 (4.1%) cases. The lowest incidence of both *U. urealyticum* and *M. hominis* was observed in the group of women with the urethral syndrome, being 19/103 (18.4%) and 2/103 (1.9%), respectively.

Of 248 women who underwent complex microbiological diagnostic procedures, 79 (31.9%) had *U. urealyticum* and 8 (3.2%) *M. hominis* infection. The coexistence of *U. urealyticum* infection with other sexually transmitted pathogens has been presented in *Tab. 2*. *U. urealyticum* infection showed most frequent coexistence with *Candida albicans* (29.1%), and less frequent with *C. trachomatis* (13.9%) and *M. hominis* infection (10.1%). *U. urealyticum* infection alone was observed in 37/79 (46.8%), and 1/8 (12.5%) patient had only *M. hominis* infection.

Tab. 3 presents the prevalence of clinical symptoms in women with only *U. urealyticum* infection. The most common symptoms were: vaginal discharge (43.3% of cases), burning and ichting of external sexual organs (18.9%), dysuria (10.8%) and hypogastric pain (10.8%). Other clinical symptoms were not so frequent. In 8.1% of the women, the course of *U. urealyticum* infection was asymptomatic. The detection frequency of *M. hominis* and *U. urealyticum* according to age is shown in *Tab. 4*. The infection was most common in women in the age range 26--30 years (29.2% for U. urealyticum and 50.0% for *M. hominis*). In women aged 31-39 years, the infection was found in 24.8% and 20%, 21-25 years in 21.1% and 25.0%, 40-49 years in 19.9%

Table 4. **Detection of *M. hominis* and *U. urealyticum* in the different age groups**

Age group (years)	*M. hominis* n (%)	*U. urealyticum* n (%)
18-20	0 (0.0)	2 (1.3)
21-25	5 (25.0)	34 (21.1)
26-30	10 (50.0)	47 (29.2)
31-39	4 (20.0)	40 (24.8)
40-49	1 (5.0)	32 (19.9)
50-59	0 (0.0)	6 (3.7)
Total	20 (100)	161(100)

and 5.0% of cases, respectively for *U. urealyticum* and *M. hominis*. In the extreme age groups 18-20 and 50-59, *M. hominis* was not detected, while *U. urealyticum* was present in 1.3% and 3.7% of the cases, respectively.

Discussion

Of the 541 study women, 161 (29.8%) had *U. urealyticum* and 20 (3.7%) *M. hominis* infection. There was a distinct disproportion in the incidence of these two mycoplasm species. *U. urealyticum* was detected in a decisive majority of infections.

Our results are very similar to those obtained by Elias et al., who in the group of 222 women in a similar age range found *U. urealyticum* in 31.8% and *M. hominis* in only 3% of the cases [5]. Schlicht et al. found a higher prevalence of *U. urealyticum*, as compared to our study, in 54% of students with abnormal urogenital findings and ureaplasmas recovered [6]. A substantially higher percentage of detected *U. urealyticum* infections than in our study may be associated with age and sexual activity of the selected group of young women. Low detection rate of *M. hominis* may be due to the fact that there were no women with bacterial vaginosis in our study group. Paavonen et al. and Shafer et al. have revealed that this pathogen is detected significantly more often in women with bacterial vaginosis than in those without BV [7,8]. The association of *M. hominis* with bacterial vaginosis has been confirmed by Keane et al., who revealed genital carriage of this microorganism in 53% of women with BV and in none without BV [9].

We found the highest percentage of positive cultures in patients of STD clinic – 59.1% and 9.1% for *U. urealyticum* and *M. hominis*, respectively. This is consistent with literature data, which indicate a high prevalence of these pathogens in the women reporting sexual risk behaviour [1,10]. Koch et al. showed that of all genital pathogens, *U. urealyticum* was cultured in the vaginal fluid of the STD patients most frequently [11].

Among infertility patients, *U. urealyticum* in cervical swabs was detected in as many as 37.2% of cases, while *M. hominis* only in 2.3%. In a study by Stray-Pedersen et al., in the cervical samples *U. urealyticum* was present in about 50% of infertile women and positive cultures from the endometrium were obtained in 26% of these women [12].

In Poland, the relationship between the incidence of mycoplasmal infections and fertility impairment in women was investigated by Elias et al. [5]. In a group of 30 infertile women they isolated *U. urealyticum* in the cervical swabs from 33.3% of the patients, while *M. hominis* was not detected at al. In the study by Rodrigueaz et al., the presence of *U. urealyticum* was related to infertility. These authors found ureaplasmal infections in 23.5% and *M. hominis* in 4.8% of the examined women [13].

In our study, the incidence rate of *U. urealyticum* infection was the third highest in patients with the symptoms of colpitis. *U. urealyticum* was detected in 35.8%, while *M. hominis* in 4.1% of the examined. Yavuzdemir et al. found similar incidence rates of *U. urealyticum* and *M. hominis* in women with vaginal discharge (33.9% and 11%, respectively) [14]. Also Sahoo et al. obtained similar results, finding *U. urealyticum* in 43% of 93 women with colpitis symptoms and detecting no *M. hominis* [15]. Di Bartolomeo et al. reported considerably higher percentages of both microorganisms (61.4% *U. urealyticum* and 16.5% *M. hominis*) [16].

In patients with cervical inflammation symptoms (with or without erosion), *U. urealyticum* was isolated in 28.7% and *M. hominis* in 3.8% of cases. Bhandari et al. detected the presence of *U. urealyticum* in 56% of chronic cervicitis women and in 38% of the cases this pathogen was the only one [17]. In the study conducted by Pisani et al., *U. urealyticum* and *M. hominis* were the most common microorganisms in women with abnormal colposcopic findings [18].

In patients with adverse pregnancy outcome, *U. urealyticum* was detected in 27.1% of cases, while *M. hominis* in 2.9%. Ye et al. found higher percentages of both species in women with spontaneous abortion due to early embryonic death. In these cases, *U. urealyticum* was found in as many as 74.1% and *M. hominis* in 27.6% [19]. Unzeitig et al. in cervical-vaginal swabs isolated *U. urealyticum* in 59% and *M. hominis* in 18% of women with spontaneous abortion [20]. Donders et al., Horowitz et al., Mahler et al. and Abele-Horn et al. observed an increased risk of miscarriage associated with genital mycoplasmal infection [21-24].

According to some authors, *M. hominis* is a major pathogen in pelvic inflammatory diseases, being detected in 10-40% of cases [25-28]. In our study, the percentage of *U. urealyticum* was considerably higher (24%) and it was in agreement with the data reported by Elias et al. who in PID patients found *U. urealyticum* in 35.5% and *M. hominis* in 3.2% of cases [5]. The results of studies conducted by Abele-Horn et al. and Miettinen in patients suffering from PID, also indicate the role of *U. urealyticum* in this clinical syndrome [24, 28].

In patients with the urethral syndrome, *U. urealyticum* was detected in 18.4%, while *M. hominis* in 1.9% of the cases. Avites and Zaragaza provided similar data [29]. Potts et al. in women with chronic urinary symptoms detected *U. urealyticum* in 45.8% and *M. hominis* in 2.1% [30]. According to Schlicht et al., in young women with abnormal urogenital symptoms *U. urealyticum* was twice as frequent as *M. hominis* [6]. Literature data and our own study results seem to confirm a significant role of *U. urealyticum* in the inflammatory states of the urinary system in women.

In our study, *U. urealyticum* infection coexisted most frequently with *C. albicans* infection (29.1%), less with *C. trachomatis* (13.9%) and *M. hominis* (10.1%). There were no women with *Neisseria gonorrheae* (*N. gonorrheae*) infection in

the study group. Belkum et al. observed a similar coexistence of sexually-transmitted infections [10]. Chinese researchers Liu et al., however, obtained different results [31]. In their study, 119 women with urogenital infection symptoms underwent complex microbiological diagnostic procedures, and 27.7% of the cases were *U. urealyticum* positive. The authors found an incidence of coinfection with other pathogens, such as *N. gonorrhoeae* 14.5%, *C. albicans* 13.5%, *M. hominis* 8.7%.

Among the most commonly reported complaints in *U. urealyticum* infected women there were: vaginal discharge – 43.3%, vulval or vaginal irritation – 18.9%, dysuria – 10.8% and lower abdominal pain – 10.8% of cases. Other ailments were much less frequent. In 8.1% of the patients the course of *U. urealyticum* infection was asymptomatic. The symptoms found to accompany *U. urealyticum* infection usually occur in genital infections of other aetiologies.

The incidence of *U. urealyticum* and *M. hominis* infections was also analysed in relation to age. The rate was higher in the age range of 26-30 years (29.2% for *U. urealyticum* and 50.0% for *M. hominis*). The results are consistent with literature data [32,33].

Conclusions

1. In the study group of women, the incidence rate of genitourinary infections due to *U. urealyticum* was considerably higher as compared to *M. hominis* infection.
2. Sexual mycoplasmal infections were most frequently reported in the group of patients of STD clinic.
3. *U. urealyticum* infection of the genitourinary system in women are usually clinically symptomatic.
4. The incidence of *U. urealyticum* and *M. hominis* infections of the female genitourinary system is distinctly correlated with age and sexual activity.
5. The diagnosis of the inflammatory states of the genitourinary system and their complications should involve tests for these pathogens, especially for *U. urealyticum*.

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Mycoplasma hominis and *Ureaplasma urealyticum* infections in male urethritis and its complications

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Abstract

Purpose: The aim of this study was to estimate the incidence of *M. hominis* and *U. urealyticum* infections among men with urethritis and its complications.

Material and methods: Material for analysis were urethral swabs and EPS. Mycoplasma IST 2 kit was applied to diagnose mycoplasmal infections. All patients were additionaly tested for *C. trachomatis*, *C. albicans* and *T. vaginalis* and Gram stain specimens were obtained to identify the presence of PMN.

Results: *U. urealyticum* was detected in 57/390 (14.6%), and *M. hominis* in 4/390 (1%) men. Exclusive *U. urealyticum* infection was found in 45 (11.5%) men, and only 2 patients had exclusive *M. hominis* infection. *U. urealyticum* infection the most frequently coexisted with *C. trachomatis* – 5 (8.8%), next with *C. albicans* – 4 (7%) and *M. hominis* – 2 (3.5%) infections. Mycoplasmal infections were the most frequently found in patients aged 30 to 39 (35.1%) diagnosed with epididymitis (29.2%). The most commonly reported symptom was dysuria.

Conclusions: *U. urealyticum* is the common pathogen among men with urethritis and its complications. The most common symptoms in *U. urealyticum* patients were: dysuria, hypogastric pains and urethrorrhoea, however, clinical symptoms are not frequently observed.

Key words: *Ureaplasma urealyticum*, *Mycoplasma hominis*, urethritis, epididymitis, prostatitis.

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Introduction

Mycoplasmas are the smallest identified free-living organisms that comprise a large group of microorganisms widespread in nature. Found in plants, animals and humans, they cause various ailments or constitute the commensal flora [1]. There are two species which are pathogenic to humans: Mycoplasma and Ureaplasma. The mycoplasmas isolated from humans tend to inhabit the respiratory and urinary tract mucosa [2]. Three species have been isolated from the surface of the genitourinary tract mucosa: *Mycoplasma hominis* (*M. hominis*), *Ureaplasma urealyticum* (*U. urealyticum*) and recently discovered *Mycoplasma genitalium* (*M. genitalium*). They are referred to as "sexual mycoplasmas", as they cause the infection via sexual contacts. Their pathogenicity is likely to be associated with the ability to adhere to epithelial cells of the genitourinary tract, to erythrocytes and spermatozoa [3]. The involvement of *M. hominis* and *U. urealyticum* in the inflammatory conditions of the male genitourinary organ still arouses numerous controversies. Their presence is associated with urethritis and its complications, such as epididymitis, prostatitis or infertility [4-6].

Aim of study

The aim of the current study was to assess the incidence of *M. hominis* and *U. urealyticum* in men with urethritis and its complications.

Material and methods

The study group consisted of 390 men aged 18-59 years (mean 38.5), with clinical symptoms of inflammation of the genitourinary tract, who were referred to the Center for Sexual Transmitted Disease Research and Diagnostics in Białystok from urological and venereological outpatient departments and from Endocrinology Outpatient Unit, Department of Gynecology and Obstetrics, University Hospital of Białystok. Patients of the latter had infertility diagnosed basing on the assessment

***Table 1*. Prevalence of *M. hominis* and *U. urealyticum* detection in studied groups of men**

Studied group	*M. hominis*		*U. urealyticum*	
	+ n (%)	– n (%)	+ n (%)	– n (%)
urethritis (n=111)	0 (0)	0 (0)	13 (11.7)	98 (88.3)
prostatitis (n=156)	0 (0)	0 (0)	13 (8.3)	143 (91.7)
epididymitis (n=41)	0 (0)	0 (0)	12 (29.3)	29 (70.7)
fortility impairment (n=37)	1 (2.7)	36 (97.3)	8 (21.6)	29 (78.4)
patients of STI clinic (n=45)	3 (6.7)	42 (93.3)	11 (24.4)	34 (75.6)

***Table 2*. Coexsistence of *U. urealyticum* infections with the other sexually transmitted pathogens (n=57)**

Pathogen	n (%)
U. urealyticum + *C. trachomatis*	5 (8.8)
U. urealyticum + *C. albicans*	4 (7)
U. urealyticum + *M. hominis*	2 (3.5)
U. urealyticum + *T. vaginalis*	0 (0)
U. urealyticum + *C. trachomatis* + *C. albicans*	1 (1.7)
U. urealyticum	45 (79)

***Table 3*. Frequency of clinical symptoms observed among men with exclusive *U. urealyticu*m infection (n=45)**

Symptoms	n (%)
dysuria	31 (68.9)
erythema of external meatus of the urethra and/or glans penis	12 (26.7)
mucous or mucopurulent discharge	9 (20)
hypogastric pain	19 (42.2)
hematospermia	5 (11.1)
painful erection	3 (6.7)
asymptomatic course	10 (22.2)

of spermatogram in accordance with WHO requirements [7]. Urethritis was identified in 111 patients, chronic prostatitis in 156, epididymitis in 41, while fertility impairment in 37. In 45 patients, the examination was performed for epidemiological reasons (casual sexual contacts).

Material for analysis included urethral swabs and EPS-expressed prostatic secretions. Mycoplasma IST 2 kit (BioMerieux) was applied to diagnose mycoplasmal infections. It enables culture, identification, indicative enumeration and antibiotic susceptibility testing (with 9 antibiotics) of *M. hominis* and *U. urealyticum*. It combines a selective culture broth with a strip containing 22 testes. This kit allows pathogen identification within 48 hours and determines the amount of bacteria, thus making differentiation possible between colonization and infection (cell count above 10^4 is the evidence of infection). The combination of three antibiotics and one antifungal agent provides selectivity, ensuring that any contamining flora present in the specimen does not affect the test. Moreover, all male patients were examined for *Chlamydia trachomatis* (*C. trachomatis*) using direct immunofluorescence method with monoclonal antibodies (Trinity Biotech), and for *Trichomonas vaginalis* (*T. vaginalis*) and *Candida albicans* (*C. albicans*) using standard culture methods (Biomed). In all the 390 patients, urethral and prostatic secretions were examined using the Gram staining procedure (direct examination) to determine the presence of polymorphonuclear leucocytes (PMN) and to exclude Neisseria gonorrhoeae infection (*N. gonorrhoeae*). Urethritis was diagnosed when PMN count was 4 or higher, while prostatitis when the count was 10 or higher in a field of vision under a light microscope at magnification of 1000 [8].

The study was approved by the Ethics Committee, Medical University of Bialystok.

Results

In the group of 390 men, *U. urealyticum* infection was found in 57 (14.6%), while *M. hominis* in 4 patients (1%).

The incidence of *M. hominis* and *U. urealyticum* according to clinical diagnosis has been presented in *Tab. 1*. *U. urealyticum* infection was most frequently observed in patients with epididymitis – 29.2%, then in men with fertility impairment – 21.6% and in venereological patients – 24.4%. *U. urealyticum* was found in 11.7% of urethritis patients and in 8.3% of prostatitis patients. *M. hominis* was identified in venereological and infertile men (6.7% and 2.7%, respectively). The remaining groups of patients were *M. hominis* negative. Our results showed a distinct dysproportion between the incidence of both mycoplasmas.

U. urealyticum was much more common than *M. hominis*.

The coexistence of *U. urealyticum* with other sexually transmitted pathogens has been shown in *Tab. 2*. Isolated *U. urealyticum* infection was observed in 45 patients, while co-infection was revealed with *C. trachomatis* (8.8%), *C. albicans* (7%) and *M. hominis* (3.5%), but not with *T. vaginalis* or *N. gonorrhoeae*. Only in one case, three pathogens: *U. urealyticum*, *C. trachomatis* and *C. albicans* were involved. Isolated *M. hominis* infection was detected only in 2 cases (0.5%).

The incidence of clinical symptoms in men infected with U. urealyticum alone has been presented in *Tab. 3*. The most common symptoms included dysuria (68.9%), hypogastric pain (42.2%) and reddening of the external meatus of the urethra and/or glans penis (26.7%). Other complaints including urethrorrhoea, haematospermia or painful penile erection were less common (20%, 11.1% and 6.7%, respectively). In 22.2% of cases, mycoplasma infections were clinically asymptomatic.

The incidence of *M. hominis* and *U. urealyticum* according to age has been presented in *Tab. 4*. The highest incidence rate

Table 4. **Incidence of *M. hominis* and *U. urealyticum* regarding age of patients**

Age	*M. hominis* n (%)	*U. urealyticum* n (%)
18-19 (n=6)	0 (0)	1 (1.7)
20-25 (n=50)	1 (25)	10 (17.5)
26-29 (n=64)	0 (0)	9 (15.8)
30-39 (n=143)	2 (50)	20 (35.1)
40-49 (n=90)	1 (25)	12 (21.1)
50-59 (n=37)	0 (0)	5 (8.8)
Total (n=390)	4 (100)	57 (100)

was noted for the age range of 30-39 years (35.1%), then 40-49 (21.1%). Negative results were the most common in the peripheral age intervals – 18-19 and 50-59 years.

Discussion

The most common infection of the lower part of the genitourinary tract in men is nongonococcal urethritis (NGU). In our NGU patients, *U. urealyticum* was found only in 11.7% of cases. According to Taylor-Robinson and Furr, mycoplasmas, especially *M. genitalium* and *U. urealyticum*, are the second common aetiological factor of this ailment, after *C. trachomatis* [9]. This has been confirmed by other researchers [10, 11]. Also Varela et al. have shown that there has been a growing incidence of *U. urealyticum*-induced urethritis in men recently [12]. *U. urealyticum* involvement in chronic NGU has been emphasized [13]. Horner et al. have demonstrated that acute urethritis is associated with *M. genitalium* and *C. trachomatis* infection, but not with *U. urealyticum* [14]. In a study by McKee et al. which involved 400 American soldiers with the symptoms of urethritis, this microorganism was isolated in 19% [15]. Chandeying et al. performed microbiological examination of urethral swabs collected from 479 students in southern Tailand [16], detecting *U. urealyticum* in 10.9% and *M. hominis* in 1.3% of the patients.

In fertility impairment, it is *U. urealyticum* that is usually involved [17,18]. We found this pathogen in men with fertility impairment in 21.6%, while *M. hominis* only in 2.7%. Unzeitig et al. emphasize the relatively high percentage of *U. urealyticum* (91%) in the sperm of sexual partners of women with secondary infertility and point at the reduced semen quality [17]. A similar role of ureaplasmas has been indicated by Taylor-Robinson and McCormack [5]. Biernat-Sudolska et al. have revealed a considerably higher incidence of U. urealyticum in patients treated for infertility (38%), who had abnormal semen patterns [19]. However, a study by Andrade-Roch indicates that routine diagnostic investigations for mycoplasmas is not clinically significant [20]. The author also emphasizes the scarcity of reports on the effect of these microorganisms on the quality of semen. However, Purvis and Christiansen suggest that *U. urealyticum* infection may play a significant role in male infertility [21], although the microorganisms can be found as commensals in the genitourinary tract. On the other hand, Rodrigues et al. have demonstrated that infertility diagnostics requires investigations for mycoplasmas, also for *C. trachomatis* and *N. gonorrhoeae*. They found a positive correlation between *U. urealyticum* infection and infertility [18].

There are very few literature reports on the significance of sexual mycoplasmas in prostatitis [22,23]. The role of atypical bacterial flora, and particularly the involvement of mycoplasmas in this ailment still remains unclear [24]. We found *U. urealyticum* in 8.3% of prostatitis patients, while *M. hominis* was not detected. Skerk et al. examined a group of 388 patients with the symptoms of chronic prostatitis [25], confirming its bacterial aetiology in 71.1% of cases, of which only 2.5% were *U. urealyticum* positive. Weidner et al., examining a group of 187 men with clinical symptoms of prostatitis [26], isolated *U. urealyticum* in as many as 103 patients (55.1%). However, these authors finally admitted that these bacteria could be responsible for prostatitis in 8.6% of cases, just like in our study group. Taylor-Robinson and McCormack emphasize the fact that the results are difficult to interpret as the microorganisms detected in the prostatic secretion may actually come from the urethra [4].

Even fewer reports are available on the mycoplasmal infections among patients with epididymitis. We found *U. urealyticum* infection in 29.9% of men with this ailment, being the highest percentage among all the study groups. All the patients were *M. hominis* negative. Eickhoff et al. detected *U. urealyticum* in 15% of their epididymitis patients, while *M. hominis* in 1.9% [27]. Joly-Guillon and Lasry believe that the type of bacteria responsible for genitourinary tract infections in males, including epididymitis, is age-dependent [28]. In under 35 year old patients sexually-transmitted bacteria prevail, including mycoplasmas, *C. trachomatis* and *N. gonorrhoeae*.

In our study, *U. urealyticum* was found in 24.4% of venereological patients. The men were frequently asymptomatic and they were referred to diagnostic examinations for epidemiological reasons. Frequent occurrence of sexual mycoplasmas (40--70%) in the lower part of the female reproductive system facilitates their transitory colonization in men due to sexual contacts. Risi and Sanders have revealed that the percentage of *U. urealyticum* in men who had more than 14 sexual partners was 56% [6]. However, whether this is colonization or infection depends on the amount of culture-grown microorganisms.

Patients with isolated *U. urealyticum* most commonly reported dysuria (68.9%) and hypogastric pain (42.2%). Every fifth man was asymptomatic.

The majority of sexual mycoplasmas - positive results were obtained in men aged 30-39 years (35.1%) and 40-49 years (21.1%). According to literature data, young and sexually active men are most frequently affected [29]. Similar results were obtained by Biernat-Sudolska et al. [19]. A slight, but distinct, reduction in age limit in our material can be due to a high number of patients with prostatitis.

Conclusions

1. *U. urealyticum* is frequently found in the genitourinary tract in men with urethritis and its complications.
2. *U. urealyticum* infection is most frequently diagnosed in men with epididymitis and venereological patients.

3. Although *U. urealyticum* infection might be clinically asymptomatic but the most common symptoms are: dysuria, hypogastric pains and urethrorrhoea.

Acknowledgement

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Orbital pseudotumor caused by a foreign body – a case report

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Abstract

The case of a patient with a foreign body in the orbit is presented. The presence of this foreign body induced an aggressively expanding pseudotumor, infiltrating the eyelids, orbital muscles and the sclera. The process of diagnosis, management and the results of treatment are described.

This case is noteworthy because of the atypical course of the disease in a patient with a foreign body following ocular injury.

Key words: foreign body, orbit, pseudotumor.

Introduction

Orbital pseudotumors represent an idiopathic, non-specific and rarely occurring disease of the orbit, with features of non-carcinomatous and non-inflammatory changes [1]. In accordance with this definition, the main condition necessary for the diagnosis of pseudotumors is the lack of any identifiable local or more general evidence which might be responsible for the growth. Hence, most authors are prepared to diagnose pseudotumors only after having excluded aetiology of an infectious or traumatic nature, those of an immunological or carcinomatous origin and general conditions that may cause changes in the orbit [2]. It is certain that pseudotumors are not a type of granulomatous or lymphatic tumor [3].

Nonetheless, immune response cells, in particular lymphocytes and different stages of local fibrosis and neovascularisation are present in histopathological specimens of pseudotumor in newly formed connective tissue. Indeed, the relative proportion of inflammatory cells with respect to collagen fibers is used to differentiate the pseudotumor into the more frequently occurring inflammatory type versus the rarer fibromatous type [3,4]. Perivascular inflammatory changes and secondary inclusion of vessels occurs rather more infrequently, which testifies to the somewhat "atypical" histological pattern of the pseudotumor [3].

The case of a patient presenting with a pseudotumor caused by a metallic foreign body and confirmed histopathologically would seem to be interesting in the context of the two, somewhat contrary opinions on the aetiology of pseudotumors, where the condition for diagnosis of the pseudotumor requires: 1) the presence or 2) the absence of an inflammatory cause.

Case report

A 40-year old male patient, a plumber by occupation, was admitted to the Ophthalmology Department 17 days after an eye injury with saw. In the external region of the orbit soft, inflammatory oedema was identified. Neither exophthalmus, nor any disturbances in eyeball movements were observed and the anterior region of the eye was normal as well. In the upper temporal, peripherial part of the fundus, a yellow, convex formation which measured 1/4 dd and containing small haemorrhages was observed, together with some restricted retinal detachment. Visual acuity on admission was 5/7, IOP=17 mmHg. The CT scan revealed the presence of an intraorbital foreign body 4 x 6 mm in size, lying peripherally to the eyeball, as well as a small, soft tissue formation of density 36-40 H, which was probably inflammatory in origin.

An attempt to remove the foreign body by surgery was undertaken, but it could not be found in the surrounding tissue which contained marked pathological changes. The surgical procedure was finally limited to the evacuation of an orbital abscess, which was further treated with the application of systemic, topi-

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Figure 1. **CT examination**

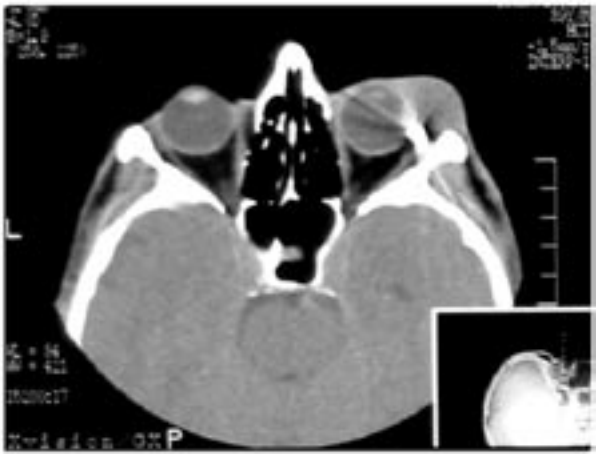


Figure 2. **Tumor removed from the orbit**

Figure 3. **Foreign body**

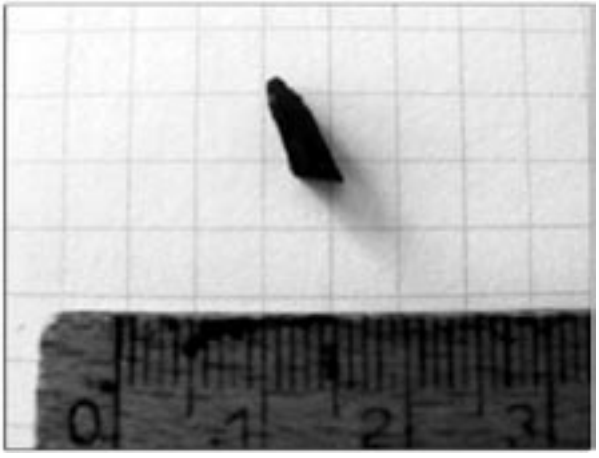
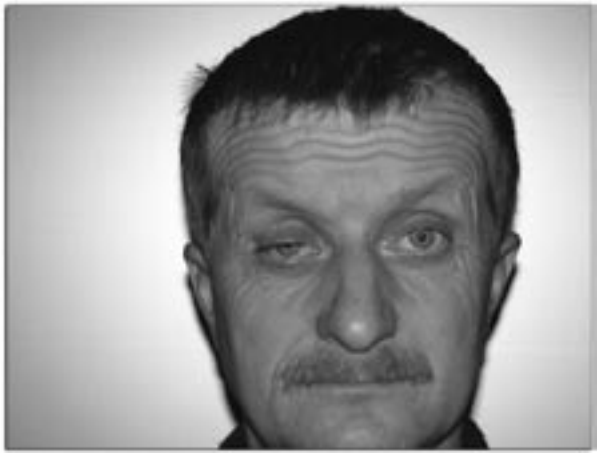


Figure 4. **Patient 4 weeks after surgical procedure**



cal antibiotics and steroids. In addition, photocoagulation of the detached retina was performed with an argon laser.

The patient was readmitted to the Ophthalmology Department after 7 months, not having attended any follow-up outpatient appointments. On admission a painless tumor of the right orbit was found. The tumor was hard, deforming the upper eye lid, 20 x 30 mm in size and restricting movements of the eyeball, which gave rise to diplopia when the patient attempted to look to the right. Visual acuity was 5/8 at this time and the ocular pressure was 24 mmHg. Computerised tomography confirmed the presence of a foreign body and also showed inflammatory, hypodensive masses, 18 x 30 mm in size (*Fig. 1*). The patient was once more qualified for the surgical removal of the pathological formation, but he definitively refused consent to the suggested treatment.

The patient was examined again after a further period of 2 months. The fibroplasias were observed to have developed aggressively in the region of the upper lid, totally closing the palpebral fissure. Visual acuity was 5/16, IOP=22 mmHg. Eyeball movement was highly restricted. Inflammatory infiltration with eosynophilic granulocytes with vessel proliferation and fibrosis were found in the BAC sample. Other diagnostic tests did not show any deviations from normal. Therapy with antibiotics and steroids did not lead to any improvement. Finally with the patient's agreement further surgical treatment was performed. During surgery, a hard tumor was dissected out, 40 x 35 x 25 mm in size, which was joined by solid, impacted connective tissue to the periosteum of the upper and lateral wall of the orbit. Fibrous adhesions were separated from the internal part of the eyelid and tarsus and also from the lateral surface of the sclera. The lateral and superior rectus muscles were not found, apparently because of tumor infiltration. A metallic foreign body, 6 x 3 x 2 mm in size was finally removed. The pathomorphological diagnosis was: *Pseudotumor inflammatorius*. On discharge from hospital, the patient's visual acuity was 5/10, IOP=12 mmHg. Right eyelid ptosis and partial restriction of eyeball movements were diagnosed. During fundus examination and ultrasonography, retinal detachment was not found (*Fig. 2*, *3*).

Follow-up examination at 4 weeks post-surgery, revealed a satisfactory cosmetic effect with regard to the position of the eyelid as well as the range of movement of the eyeball, which was only minimally restricted. Visual acuity was 5/8, IOP=14 mmHg. Slight diplopia occurred only when patient looked to the right (*Fig. 4*).

Discussion

The definition of pseudotumors presented in the introduction refers only to those changes which may be described as an idiopathic inflammation of the orbital tissues. Nevertheless, many authors point to the role of infections, autoimmune diseases and anomalous wound healing as potential causes for the development of pseudotumors [3,5,6]. It seems very probable that in the case presented here, the presence of a foreign body was the direct reason for the occurrence and aggressive growth of the tumor [7,8].

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Met-enkephalin in the liver as a marker of hepatocellular damage in chronic viral hepatitis type B and C

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Abstract

Purpose: The aim of the study was to assess the liver Met-enkephalin concentration in chronic viral hepatitis type B and C as well as in liver cirrhosis in order to estimate the role of opioid system in pathogenesis of liver disease.

Material and methods: The concentration of Met-enkephalin was examined in liver tissue of 103 consecutive patients with chronic hepatitis type B and C. Control group consisted of uninfected patients. Met-enkephalin concentration was analyzed in relation to the degree of hepatic necroinflammatory activity and the extent of fibrosis estimated by histopathological examination of liver bioptates and compared to such parameters as age, sex and concomitant diseases.

Results: Significant differences in Met-enkephalin concentration were found between cases with advanced fibrosis (stage 3 and 4 acc. to Batts and Ludwig classification) and cases with fibrosis classified as stage 2 ($p<0.05$). Met-enkephalin concentration was higher in HCV infected patients in comparison to HBV infected patients ($p<0.05$) and uninfected controls ($0.05<p<0.1$). There wasn't found any correlation between Met-enkephalin level and necroinflammatory activity in the liver, age, sex and concomitant diseases.

Conclusions: Met-enkephalin concentration measurement in the liver tissue seams to be a useful method for differentiation of stage 2 from stages 3 and 4 of severe liver fibrosis. There is increased concentration of Met-enkephalin in liver tissue in HCV infected patients in comparison to HBV infected or uninfected individuals. The degree of necroinflammatory activity in the liver as well as sex and age of patients with chronic hepatitis do not correlate with changes in opioid system.

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Key words: enkephalins, inflammation, fibrosis, chronic hepatitis, HBV, HCV.

Introduction

Many of endogenic opioid substances derived from precursor proteins as proopiomelanocortine, proenkephaline and prodynorphine have been described. Most of these substances are composed of five amino acid peptides Leu- or Met-enkephalin. There are several types of receptors (with possible subclasses) which can bind them. Various specificity of peptide-receptor binding, different agonist and antagonistic effects in different tissues with possible cross reactions make the system, participating in multiple human systems regulation, extremely complicated.

The alterations of the opioid system in patients with severe liver diseases have been described. Some reports [1,2] showed elevated Met-enkephalin level in serum in patients with acute liver damage. The level was several times higher then in control group and decreased subsequently to normalization of aminotransferases. It was shown that in patients with decompensed liver cirrhosis and ascites [3,4] Met-enkephalin level was several times higher then in patients with cirrhosis but without ascites. It is possible that small opioid peptides play an important role in pathogenesis of ascites [3]. It was reflected by their blood vessels dilatation ability [5-7] and the presence of Met-enkephalin receptors in visceral blood vessels [8]. Met-enkephalin concentration is the highest in patients with liver cirrhosis and ascites as well as cholestatic liver diseases [2]. Enkephalins elevated in serum may cross blood-brain barrier [9,10] and cause cerebral dependence – naloxon reversible analgesia [11]. It may be responsible for increased patients' susceptibility to morphine [12]. The administration of opioid antagonists in cirrhotic patients can produce an effect which is similar to the withdrawal syndrome observed in opiate drug-addicts [13,14].

Itching of the skin in cholestatic syndromes become less intense after nalmefan or nalokson, this suggests the role of

Table 1. **Characteristic of patients**

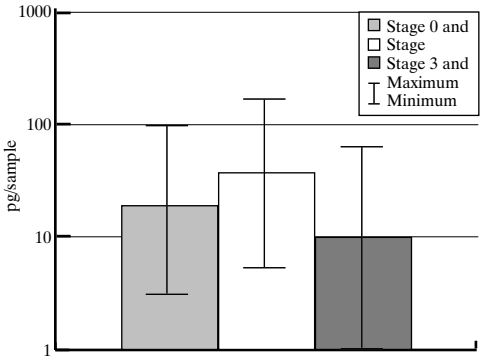
Women	Men	Mean age	HCV infection	HBV infection	Uninfected	Additional diseases
28 (27%)	75 (74%)	47	26 (25%)	54 (53%)	23 (22%)	28 (28%)

opioid system in its pathogenesis. This hypothesis is supported by observation that itching may be induced by morphine and other opioids [15].

Opioid system is thought to act as a link between neuroendocrine and immune systems [16]. Immunocompetent cells are both the source [17] and target for opioids through δ, μ, κ receptors [18]. Opioids released from immunocytes in inflammatory infiltrates can produce analgesia [19]. Moreover, it was shown in experiments *in vitro* that opioid peptides activate or inhibit lymphocytes and macrophages [20-23].

Currently available data on the effect of opioids on the immune system, encouraged us to make the hypothesis that Met-enkephalin might be the important cytokine regulating inflammatory process. The second hypothesis we have tried to verify are postulated relations between hepatic opioid system and process of hepatic fibrosis as well as chronic viral hepatitis type B and C.

Figure 1. **Met-enkephalin concentration in groups of patients according to degree of fibrosis. The results are presented in log scale**



Material and methods

Thick-needle biopsy samples of liver tissue were obtained from 103 consecutive patients with chronic HBV and HCV infected (*Tab. 1*) and from uninfected controls (biopsies were performed because of some other diagnostic indications).

Liver biopsies were performed with local anesthesia, according to Menghini's method with 1.6 mm needles. Informed written consents were obtained from all patients and the study protocol was approved by the local ethical committee. A small part from 2 mm long piece of a liver tissue was used in this study and remaining portion was examined histologically.

The liver tissue was taken to 500 μl of 0.5 N HCl and kept at -70°C until further processing. Tissues were homogenized and centrifuged for 15 min, 1500 rpm at 4°C. Supernatant (200 μl) was neutralized with 2 ml of 0.06 M phosphate buffer (pH 10.2). Native enkephalin was purified on Porapak columns with 250 mg of Porapak (Waters, 100-120 mesh) in 3 ml of absolute ethanol. Porapak slurry was prepared by degassing overnight 25 g of this material in 350 ml of absolute ethanol. Shortly before applying the samples, columns were equilibrated with 6 ml of redistilled water. Loaded column were washed with 6 ml of redistilled water and enkephalin was eluted with 3 ml of absolute ethanol, and then lyophilized by 16 h (Heto Holten). Lyophilized samples were reconstituted with 100 μl of 0.06 M phosphate buffer (pH 6.5) containing 0.2% bovine serum albumin. 50 μl of specific antiserum 18R2 diluted 1:10000 and 50 μl of 125-I-Met-enkephalin (~1500 cpm) were added and samples were incubated for 24 h at 4°C. After incubation 50 μl of 1% rabbit gamma globulin as second antibody was added and further incubated for 30 minutes at 4°C. Separation of bound from free complex was performed by adding 250 μl of 25% PEG. After 30 minutes of incubation, samples were centrifuged at 2000 rpm at 4°C for 30 minutes, supernatant was discarded, and pellets were counted in gamma-counter (LKB). The results were presented as pg per sample and pg per 1 g of total supernatant protein, according to Exton method.

The liver tissue was examined histologically with hematoxylin and eosin, Masson trichrome for collagen and Gomori for argentophilic fibers stainings. Activity of necroinflammatory process and degree of fibrosis were evaluated according to semi-quantitative five-degree Batt-Ludwig's scale [24].

Statistical analysis

Randomized group of 103 patients was statistically analyzed according to severity of fibrosis, HBV or HCV infections, activity of inflammation, serum ALT level, sex, age and concomitant diseases.

The data were not normally distributed and therefore non-parametric statistical analysis was applied using the Mann-Whitney U-test. Wilcoxon's test based on differences in Met-enkephalin levels between different determinations of the peptide in the same bioptate was done as the evaluation of the method involved, p value less then 0.05 was considered significant.

Results

Statistically significant differences were found between cases of moderate fibrosis assessed as stage 2 and advanced stages classified as close to cirrhosis or cirrhosis (*Fig. 1*). Significant differences were found between HCV and HBV infected patients ($p<0.05$) as well as between HCV infected and uninfected patients ($0.05<p<0.1$) (*Fig. 2*).

Figure 2. **Met-enkephalin concentration in groups of patients infected with HBV, HCV and uninfected controls. The results are presented in log scale**

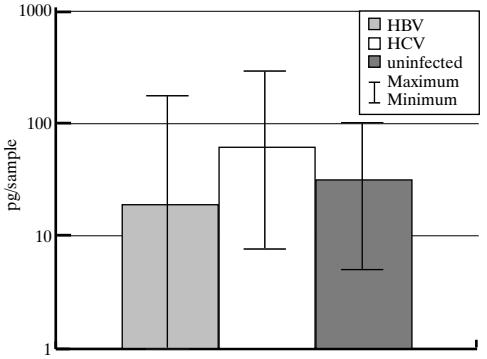
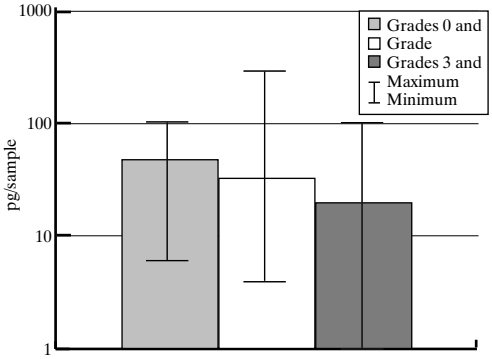


Figure 3. **Met-enkephalin concentration in groups of patients according to degree of necroinflammatory process (grades 0 to 4 acc. to Batts-Ludwig scale). The results are presented in log scale**



No correlation was found between Met-enkephalin level and necroinflammatory activity (*Fig. 3*), intralobular or portal inflammatory activity and serum ALT level.

Statistical analysis revealed no correlation between opioid level in liver tissue and such attributes as age, sex and concomitant diseases.

Met-enkephalin concentration of randomly selected samples was re-assessed in order to evaluate accuracy and repeatability of diagnostic method. Using non-parametric Wilcoxon's test, no statistically significant differences were found between obtained results. Thus, repeatability of involved method seems to be satisfactory.

Spearman's rank correlation coefficient for Met-enkephalin in bioptate and per 1 g of sample was 0.7344, which was statistically significant ($p<0.01$). It means that Met-enkephalin assessment in biopsy specimen could be used to estimate Met-enkephalin concentration in 1 g of bioptate proteins.

Discussion

Liver tissue in patients with chronic hepatitis consists of hepatocytes which are infiltrated by immunocompetent cells. These cells are responsible for necroinflammatory process which reflects activity of immune reactions. Well documented relations between opioides and immunological system [16,17] suggests possibility of correlation between altered Met-enkephalin concentration in liver tissue and severity and origin of hepatitis. This study showed a decrease of Met-enkephalin concentration in patients with the highest necroinflammatory activity in the liver. Increased Met-enkephalin concentration in liver was found in patients with chronic viral hepatitis type C in comparison to patients with HBV infection and control group. Liver damage due to HCV and HBV is characterized by immunologic reactions and differences in direct pathogenic effects of viruses can be observed. HCV, in contrast to HBV, stimulates autoimmune processes [25,26] and differ in mechanism of humoral and cellular immunity. The core proteins of HCV participate in hepatocyte damage but HBV is not directly cytopathogenic and reactions in liver tissue are caused by immunologic phenomena [27]. It seems to be possible that HCV directly affect the opioid concentration in cells, as we can see in HIV infected patients. In HIV infected the increase of β-endorphine was reported as a consequence of infection of susceptible cell [28]. However, it is impossible to determine which mechanism is responsible for observed alterations in liver enkephalin system.

The observed changes in Met-enkephalin concentration in patients with advanced liver fibrosis can be helpful in differentiation of patients with moderate fibrosis and classified as close to cirrhosis or cirrhosis. Liver cirrhosis leads to profound changes in opioid system reflected by increase in serum opioids, syndrome of chronic central opioid receptor stimulation and hypothetical participation in pathogenesis of ascites [3]. In patients suffering from primary biliary cirrhosis (PBC), serum Met-enkephalin is negative prognostic factor, its increased levels correlate with decreased survival rate [29]. Some authors [30] described significantly higher levels of serum Met-enkephalin in stages 3 and 4 in comparison to stages 1 and 2 of PBC. Decreased Met-enkephalin level in the liver with advanced fibrosis observed in our study can be caused by the same mechanism as in PBC. Low hepatic Met-enkephalin concentration in the most severe cases of fibrosis corresponds to other reports of normal enzymatic enkephalin degradation systems in liver tissue [31]. It is well known that the liver uptake of small biologically active proteins such as opioids plays an important role in clearing them from circulation [32]. Defect of non-endocytal energy dependent liver uptake may be a reason of Met-enkephalin reduction in the liver with the most advanced fibrosis, and serum Met-enkephalin elevation, which was widely reported in literature [3,4,30]. Proenkephalin together with its deriving products contribute to cellular proliferation and differentiation [33]. Liver cirrhosis is defined as progressive fibrosis with hepatocyte degeneration and parallel process of tissue regeneration. Liver Met-enkephalin may play auto- or paracrinic regulatory role in cell proliferation and differentiation. Decreased liver Met-enkephalin concentration may reflect progressive deterioration of the process of liver tissue regeneration.

In conclusion, Met-enkephalin concentrations measurement is not effective in distinguishing between a degree of necroinflammatory activity evaluated by liver histology and by serum

ALT. Decreased Met-enkephalin concentration in the liver with advanced fibrosis as well as increased level in patients with HCV infection suggest relationship between opioid peptides and both process of fibrosis and origin of liver damage.

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Serum homocysteine, folate, vitamin B_{12} and total antioxidant status in vegetarian children

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Abstract

Purpose: The results of several studies point to the positive role of vegetarian diets in reducing the risk of diabetes, some cancers and cardiovascular diseases. However, exclusion of animal products in vegetarian diets may affect the cobalamin status and cause an elevation of the plasma homocysteine level.

The aim of this study was to assess the effect of vegetarian diets on serum concentrations of homocysteine, folate, vitamin B_{12} and total antioxidant status (TAS) in children.

Material and methods: The study included 32 vegetarians (including 5 vegans), age 2-10 years. Dietary constituents were analyzed using a local nutritional programme. Serum homocysteine, folate and vitamin B_{12} were determined with fluorescence and chemiluminescence immunoassays. The concentration of TAS was measured by a colorimetric method.

Results: Average daily energy intake and the percentage of energy from protein, fat and carbohydrates in the diets of the studied children were just above or similar to the recommended amounts. It could be shown that vegetarian diets contain high concentrations of folate. In vegan diets it even exceeds the recommended dietary allowance. Mean daily intake of vitamin B_{12} in the studied diets was adequate but in vegans was below the recommended range. The serum concentrations of homocysteine, folate, vitamin B_{12} and TAS in vegetarian children remained within the physiological range.

Conclusions: The presented data indicate that vegetarian children, contrary to adults, have enough vitamin B_{12} in their diet (excluding vegans) and normal serum concentrations of homocysteine, folate and vitamin B_{12}. Therefore, in order to prevent deficiencies in the future, close monitoring of vegetarian children (especially on a vegan diet) is important to make sure that they receive adequate quantities of nutrients needed for healthy growth.

Key words: homocysteine, folate, vitamin B_{12}, vegetarian diets, children.

Introduction

The results of several studies show important benefits of vegetarian diets and a relation with reduced risk for such diseases as diabetes, obesity, heart diseases and several types of cancer [1-3]. However, exclusion of animal products in vegetarian diets may affect the cobalamin (vitamin B_{12}) status and cause an elevation of the plasma homocysteine (Hcy) level [4,5]. The predominant consumption of protein of plant origin in this kind of diets shifts homocysteine to the remethylation pathway, which requires vitamin B_{12} as a cofactor and methyltetrahydrofolate as a substrate. In vegetarian diets the intake of folic acid exceeds the recommended dietary allowance, whereas the intake of vitamin B_{12} is inadequate or even absent [6].

Adult vegetarians are at risk of developing hyperhomocysteinemia, which has been recognized as an independent cardiovascular risk factor. It is hypothesized that Hcy alters endothelial and smooth muscle cell functions by generating reactive oxygen species. The resulting increase in oxidative stress diminishes antioxidative capacity, which increases the risk for atherosclerotic vessel diseases in these subjects. Dietary folate deficiency causes insufficient formation of 5-methyltetrahydrofolate, which is needed as a donor of methyl-group in the remethylation of Hcy to methionine. Vitamin deficiencies (B_{12} and folate), enzyme mutations with partial loss of enzymatic activities (cystathionine-β-synthase, methionine synthase, methylentetrahydrofolate reductase polymorphisms), and renal insufficiency may produce moderate hyperhomocysteinemia (>15 μmol/L) [4,7].

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Table 1. **Average daily energy and nutrient intakes of vegetarian children compared to recommended daily intake**

	Daily intake	Recommended daily intake
Energy values (kcal)	1426.5±440.4	1300.0-2500.0
Energy from protein (%)	11.9±2.4	12.0-14.0
Energy from fat (%)	32.3±4.9	32.0
Energy from carbohydrates (%)	55.8±5.6	56.0-58.0
Folate (μg)	195.7±78.0	50.0-150.0
Vitamin B_{12} (μg)	1.6±1.3	1.0-2.0

Data are presented as mean values ±SD

Table 2. **Serum concentrations of lipids, homocysteine, folate, vitamin B_{12} and TAS in vegetarian children**

	Vegetarian children	Reference values
TC (mg/dL)	155.1±25.8	<170
HDL-C (mg/dL)	53.7±8.3	52-72
LDL-C (mg/dL)	91.0±24.5	<110
TG (mg/dL)	80.4±58.4	50-100
Homocysteine (μmol/L)	6.1±1.2	<8.0
Folate (ng/mL)	12.8±3.4	4.2-19.9
Vitamin B_{12} (pg/mL)	548.6±144.4	240-900
TAS (mmol/L)	1.20±0.1	1.16-1.40[25]

Data are presented as mean values ±SD and ranges

Hyperhomocysteinemia and decreased total antioxidant status (TAS) may partly counteract the beneficial lifestyle of vegetarians. No systemic studies have been carried out on homocysteine and TAS status in children on vegetarian diet.

The aim of this study was to assess the effect of vegetarian diets on serum concentrations of homocysteine, folate, vitamin B_{12} and total antioxidant status in children.

Material and methods

The study included 32 vegetarian children (14 girls, 18 boys) aged 6.5±4.2 years who had been referred to the Department of Nutrition of the Institute of Mother and Child (Warsaw) for dietary consultation. The principal difference among various vegetarian diets was the extent to which certain products were avoided. In this tested group there were:

– lacto-ovovegetarians (n=21), who did not consume meat, poultry or fish, but ate eggs and dairy products,

– lacto vegetarians (n=1), who excluded eggs,

– ovovegetarians (n=5), who ate eggs, but excluded milk products,

– vegans (n=5), who excluded all foods of animal origin.

This research was approved by the institutional review board and informed consent was obtained from parents of the examined children. Dietary constituents were analyzed using the nutritional programme Dietetyk2® (National Food and Nutrition Institute, Warsaw) and completed with supplementation data.

Venous blood samples were obtained after overnight fast. Serum was prepared by centrifugation at 1000 x g at 4°C and total cholesterol (TC), high-density lipoprotein HDL (HDL-C), low-density lipoprotein LDL (LDL-C) and triglycerides (TG) concentrations were determined enzymatically with kits from Bio-Merieux (France) using Cobas Mira analyzer (Roche, Switzerland). Remaining serum samples were frozen and collected for other analyses. Total Hcy was measured with a fluorescence polarization immunoassay on IMX analyzer (Abbott, USA). Folate and vitamin B_{12} were determined with a chemiluminescence immunoassay (Elecsys, Roche, Switzerland). TAS was measured by a colorimetric method using kits from Randox Laboratories Ltd (GB).

Results

Tab. 1 shows the average daily energy and nutrients intakes of vegetarian children including the intake of folate and vitamin $B_{12,}$ compared to nutrition recommendations. Mean daily energy intake was 1426.5±440.4 kcal and the percentage of energy from protein was 11.9±2.4, from fat 32.3±4.9 and from carbohydrates 55.8±5.6. These amounts were just above or similar to the lower limit of the recommended values. Moreover, vegetarians have a high intake of folate (195.7±78.0 μg/day). Mean daily intake of vitamin B_{12} was in the reference range (1.6±1.3 μg/day), but in 9 individuals (including all the vegans) was below the recommended values (<1 μg/day).

In vegetarian children serum concentrations of total cholesterol and LDL-cholesterol levels (*Tab. 2*) were low but in the physiological range. Triglycerides concentrations were in the middle, whereas HDL-cholesterol were close to the lower limit of the reference range. The mean serum concentrations of homocysteine (6.1±1.2 μmol/L), folate (12.8±3.4 ng/L) and vitamin B_{12} (548.6±144.4 pg/mL) were in the physiological range. Total antioxidant status in vegetarians was in the range of 1.16-1.40 mmol/L. Concentrations of TAS below the lowest value of omnivorous children were found only in 2 vegetarians.

Discussion

According to present knowledge there are several positive as well as negative consequences of vegetarianism on health status [1-3,5]. The risk of dietary inadequacy increases with the number and degree of restrictions on the food groups that are consumed, willingness to use fortified foods or nutrient supplements, and to accept medical advice. The principal difference among various vegetarian diets is the extent to which animal products are avoided. In this respect the lacto-ovovegetarian diet being the most permissive and the vegan diet the most restrictive.

Vegetarian diets in children can be healthy only, if they are well balanced and a variety of foods is consumed. It is also important to be sure that all nutrients are consumed at appropriate levels [3,8,9]. Adequate energy intake is very important for children and adolescents, because growth and development

are the most intensive at these age spans. Vegetarians usually consume less total protein than omnivores but their intakes are usually satisfactory if energy intakes are adequate. Protein quality is also of potential concern, because plant proteins are limited in some amino acids (lysine, cysteine, tryptophan, methionine) [10]. Most vegetarian diets are lower in fat, saturated fat but higher in polyunsaturated and monounsaturated fats than non-vegetarian ones. Intakes of carbohydrates, especially complex carbohydrates and dietary fiber, tend to be higher among vegetarians than among omnivores, and more in line with dietary recommendations [3,9].

Our results indicate that in the tested vegetarian children the mean daily energy intake and the percentage of energy from protein, fat and carbohydrates were similar or just above the lower limit of the recommended values.

In general vegetarians have relatively low serum cholesterol, lipoproteins and triglycerides concentrations [11-13]. However, recent studies have shown higher serum concentrations of homocysteine in vegetarians than in omnivores. The Hcy concentration increases as the vegetarian diet becomes more restrictive and peaks in vegans [4,7,14-17]. Hermann et al. [7] and Krajcovicowa-Kudlackova et al. [18] observed that about 20-30% young vegetarian had moderate hyperhomocysteinemia (>15 μmol/L).

High Hcy occurrence in children is not yet fully recognized. Our previous results, similar to the studies of other, indicated that Hcy concentration in healthy omnivorous children were half of that reported in adults [19,20]. In the presented study concerning children on vegeterian diets, we have confirmed these conclusions and have observed mean values of homocysteine in children that are lower by 40% compared to adult vegetarians.

Elevated serum homocysteine, caused by its prooxidative activity might be associated with non-efficient antioxidant protection and results in lipid peroxidation [21,22]. We have previously shown that mean serum concentrations of antioxidant vitamins A and E in prepubertal vegetarian children were statistically lower as compared to those in non-vegetarians but comparable to the reference range [23]. Total antioxidant status (TAS) values represent a mixed antioxidant capacity contributed among other compounds mainly to vitamin C, vitamin E, vitamin A and β-carotene [24]. In our group of prepubertal vegetarian children mean values of TAS were similar and in the same range as reported by others in omnivorous children and in adult vegetarians [7,25,26].

Ullegaddi et al. [22] observed that therapy with antioxidants and B-group vitamins inhibited an effect of elevated total plasma homocysteine. Several studies reported that mild hyperhomocysteinemia in vegetarians could be a consequence of vitamin B_{12} deficiency [18,27]. Vegetarian diets are typically high in folic acid because of high intakes of fruits and vegetables but are low in vitamin B_{12}, which is found in its most bioavailable form only in meat [17,28]. Vitamin B_{12} deficiency may take years to develop because the body is able to store sufficient quantities of this vitamin (in vegetarians mainly from vitamin supplementation). This is a problem not only because megaloblastic anemia may develop, but also nerve cells tend to be depleted in $B_{12.}$ Another problem is that high folate level can mask this kind of anemia and B_{12} deficiency may not be detected until after the onset of neurological symptoms.

In the group of studied children high intake of folate and normal mean daily intake of vitamin B_{12} was observed. Only in vegan children the intake of folate was above 200 μg/day and vitamin B_{12} below the recommended values. In the serum of all tested children concentrations of folate and vitamin B_{12} were in the middle of the reference range.

Therefore, it seems to be important to closely monitor vegetarian children to make sure that they receive adequate quantities of nutrients needed for healthy growth. It is also necessary to look for negative consequences of these diets, such as hyperhomocysteinemia and deficient total antioxidant status (TAS), which may partly counteract the beneficial lifestyle of vegetarians.

Conclusions

The presented results indicate that vegetarian prepubertal children, contrary to adults, have enough vitamin B_{12} (excluding vegans) in their diet and normal serum concentrations of homocysteine, folate and cobalamin. Therefore, in order to prevent future deficiencies, close monitoring of vegetarian children (especially on a vegan diet) is important to make sure that they receive adequate quantities of nutrients needed for healthy growth.

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Osteoprotegerin and C-telopeptide of type I collagen in polish healthy children and adolescents

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Abstract

Purpose: Most metabolic bone diseases are characterized by a disturbances in bone resorption, therefore biochemical markers concerning this process are of special interest. Recently, the novel cytokine osteoprotegerin (OPG), belonging to the tumor necrosis factor receptor family has been established as an endogenous inhibitor of osteoclastogenesis and resorption process. In addition serum C-telopeptide of type I collagen (s-CTX) is one of the resorption markers released into circulation as a result of the osteoclast mediated degradation of type I collagen. However, a clinical application of OPG and s-CTX in children may be difficult by less information of suitable reference data in relation to age, race and sex. The aim of our study was to invastigate serum concentrations of both markers in polish healthy children and adolescents.

Material and methods: We examined 102 healthy children and adolescents in 6-24 years of age, divided on prepubertal, pubertal and postpubertal groups. OPG and s-CTX were determined by ELISA kits from Biomedica (Austria) and Osteometer (Denmark) respectively.

Results: The highest mean values of OPG were in prepubertal girls (4.64±0.57 pmol/L) and boys (4.28±0.86 pmol/L). Next, in older children and adolescents gradually decreased of OPG concentration was observed. We also obtained the decreased of s-CTX concentration in studied children except these in pubertal period. Generally, we obtained significant positive correlation between OPG and s-CTX in all observed groups ($n=102$, $r=0.653$; $p<0.0001$).

Conclusions: We report the age-related decrease in circulating endogenous OPG during childhood and adolescence. Serum OPG concentration in postpubertal period may be similar to those presented in young adults. Prospective studies are needed to investigate the influence of OPG on bone metabolism in children.

Key words: osteoprotegerin, s-CTX, bone resorption, children, adolescents.

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Introduction

Biochemical bone markers can provide as a valuable non-invasive tool in the management of metabolic bone diseases [1,2]. They are available to assess both bone formation and bone resorption process. Because most metabolic bone diseases are characterized by disturbances in bone resorption, biochemical markers concerning this process are of special interest [3,4]. Recently, the novel cytokine osteoprotegerin (OPG), belonging to the tumor necrosis factor receptor family has been established as an endogenous inhibitor of osteoclastogenesis and resorption process. OPG binding to RANKL (receptor activator of nuclear factor kappaB ligand) and blocking its interaction with RANK (receptor activator of nuclear factor kappaB) inhibits the proliferation, differentiation, survival and fusion of osteoclastic precursor cells and promotes osteoclasts apoptosis [5,6]. Alternation in this system could form the basis of bone diseases in osteoporosis, renal osteodystrophy, rheumatoid arthritis, Cushing's disease, and human immunodeficiency virus patients [7-9]. Physiologically, the OPG levels demonstrated a positive correlation with age in both sexes. However, these results concerned adult cohorts and ageing women and men [10-11]. Data in females and males younger than 50 years of age show a low variability of serum OPG levels, whereas in accordance with many authors, greater variability was seen in the elderly [5,12]. A steep increase of OPG in females at the sixth decade and in males later at the seventh decade were observed [11].

Bone consists of a calcified organic matrix, which is composed of 90% type I collagen [13]. During bone resorption, molecule of type I collagen is degraded, and small fragments are liberated into the blood-stream. The amino acid sequence EKAHDGGR found in the C-terminal telopeptides of the α1 chain of type I collagen (CTX), which can undergo β-isomerization, has proven to be a sensitive marker of bone resorption [14]. Higher serum-CTX (s-CTX) levels are associated with lower bone mineral density values in Crohn's disease and postmenopausal osteoporosis [15,16]. S-CTX is associated also with the severity of radiographic findings in patients with rheumatoid arthritis [17]. Clinically, it has been evaluated mainly in postmenopausal women treated with bisphosphonates and hormone replacement therapy [18]. In children, normal serum concentrations of CTX were documented better than OPG. Previously studies shown that serum CTX values reflects the pediatric growth curve similar to patterns observed for other bone formation and resorption markers [19,20].

However, a clinical application of OPG and s-CTX in children may be difficult by less information of suitable reference data in relation to age, race and sex. The aim of our study was to investigate serum concentrations of OPG and CTX in polish healthy children and adolescents.

Material and methods

Our study group consisted of 102 healthy children and adolescents (52 girls and 50 boys) 6-24 years of age who had been referred to Institute of Mother and Child (Warsaw). Children were divided on subgroups: prepubertal (girls 6-8 y; boys 6-10 y), pubertal (girls 9-13 y; boys 11-15 y) and postpubertal (girls 14--24 y; boys 16-24 y). All individuals showed normal physical development and had no diseases that could affect bone metabolism. None of them was receiving any medication. Informed consent was obtained from the children's parent or from subjects who were 18 years of age. This study had been approved by the Ethics Committee of Institute of Mother and Child.

Non-fasting blood samples were obtained between 8.00 and 9.30. Blood was centrifuged at 1000 x g for 10 min, serum was separated and stored at – 20°C until assay. Osteoprotegerin was determined by enzyme immunoassay (Biomedica, Austria), in which OPG present in the sample, binds to the precoated capture antibody and forms a sandwich with the detection antibody. The intra- and interassay imprecision are 10% and 7% at 5.53 pmol/L.

The s-CTX concentration was measured using Serum Cross-Laps One Step ELISA assay (Osteometer, Biotech, Denmark). This method based on highly specific monoclonal antibody against a β-aspartate isomerized form of the sequence EKAHD--β-GGR derived from the C-terminal telopeptide region of the type I collagen α1-chain. According to manufacturer, the intra- and interassay imprecision (CVs) are 4.9% and 6.6% at 434 ng/L.

All data were compared by Student's t-test. Pearson correlation was computed between s-CTX and OPG concentration and age of studied children. Differences were regarded as statistically significant at $p<0.05$.

Results

Tab. 1 shows the OPG and s-CTX values of studied groups of children in relation to age and sex. The highest mean values of OPG were in girls (4.64±0.57 pmol/L) and boys (4.28±0.86 pmol/L) during prepubertal period. Next, in older children and adolescents gradually decreased of OPG concentration was observed. The significantly lower mean values were obtained in postpubertal girls (3.55±0.70 pmol/L) and boys (3.56±0.36 pmol/L) in comparison to prepubertal and pubertal period (girls, $p<0.05$; boys, $p<0.01$). None statistically significant differences in OPG concentration between girls and boys in particular periods of life were observed. The negative correlation between age and OPG was significant in postpubertal girls, in all groups of boys and girls, and in all studied children (*Tab. 2*).

Mean values of s-CTX were similar in prepubertal children: 2029±361 ng/L for girls, 1883±374 ng/L for boys (*Tab. 1*). The s-CTX levels increased slightly (about 10%) in girls and significantly (about 20%) in boys during puberty to the mean values 2266±368 ng/L and 2281±474 ng/L ($p<0.01$) respectively. After puberty, when bone mineral consolidation occurs, level of s-CTX decreased about 3-fold in girls and 2-fold in boys as compared to the pubertal children. In general, girls showed decreased postpubertal values of s-CTX about 2 years earlier than boys, reflecting the earlier completion of puberty. None statistically significant differences in s-CTX concentration between girls and boys in particular periods of life were observed. The negative correlation between age and s-CTX was significant in all tested groups except prepubertal boys and pubertal children (*Tab. 2*). We also observed significant positive correlation between OPG and s-CTX in group of all tested children ($n=102$, $r=0.653$; $p<0.0001$).

Discussion

We have shown in healthy children and adolescents, that serum OPG levels decreases with age without a gender difference. Moreover, we found a positive association between serum OPG and s-CTX in studied group.

For the first time Buzi F et al. [21] compared serum OPG concentration in a group of 46 normal children (1-14 years old). These authors obtained mean value of OPG 4.05 ± 1.63 pmol/L with no difference between males and females. In children 4-14 years old the level of this marker was similar to those present in young adult men 3.55±0.97 pmol/L [12]. However, our results concerning children 6-14 years old shown higher value for OPG (4.36±0.70 pmol/L) than was reported by Buzi et al. [21]. Moreover, OPG concentrations obtained by us in these prepubertal and pubertal children were also significantly higher ($p<0.0001$) than in adolescents 15-24 years old (4.36±0.70 pmol/L vs 3.48±0.36 pmol/L). Therefore, we think that OPG concentration in postpubertal period may be rather similar to those presented in young adults [12].

High level of OPG was observed in infancy, a decrease to steady levels in childhood and adulthood until 45 years and a further progressive increase until senescence [11]. In accordance with Buzi et al. [21] we obtained an inverse correlation

***Table 1*. The concentration of OPG and s-CTX in healthy children and adolescents.**

Gender	Prepubertal groups		Pubertal groups		Postpubertal groups	
	Girls n=14	Boys n=21	Girls n=17	Boys n=19	Girls n=21	Boys n=10
OPG (pmol/L)	4.64±0.57	4.28±0.86	4.28±0.67	4.20±0.48	3.55±0.70•	3.56±0.36••
s-CTX (ng/L)	2029±361	1883±374	2266±368	2281±474*	821±447***	1069±552**

Data are shown as mean value ±SD; •postpubertal girls vs prepubertal and pubertal girls, $p<0.05$; •• postpubertal boys vs prepubertal and pubertal boys, $p<0.01$; *pubertal boys vs prepubertal boys, $p<0.01$; **postpubertal boys vs prepubertal, $p<0.001$ and pubertal boys, $p<0.0001$; ***postpubertal girls vs prepubertal and pubertal girls, $p<0.0001$

***Table 2*. Correlation between age and OPG or s-CTX**

Group of children	OPG		s-CTX	
	r	p	r	p
prepubertal girls n=14	-0.216	NS	-0.574	p<0.01
pubertal girls n=17	0.002	NS	0.042	NS
postpubertal girls n=21	-0.461	p<0.05	-0.710	p<0.0001
all girls n=52	-0.649	p<0.0001	-0.786	p<0.0001
prepubertal boys n=21	-0.073	NS	-0.385	NS
pubertal boys n=19	-0.034	NS	0.140	NS
postpubertal boys n=10	-0.466	NS	-0.711	p<0.02
all boys n=50	-0.412	p<0.01	-0.390	p<0.01
all children n=102	-0.517	p<0.0001	-0.619	p<0.0001

of OPG with age. Moreover it seems, that OPG don't reflect exactly the pediatric growth curve. The trend of this marker levels during puberty period appears to be a little different than bone resorption and formation markers. Contrary to OPG we observed the increased (but not very high) of s-CTX concentration leading to a peak in pubertal stage. It is well recognized that bone mass increases with age from infancy to adolescence and that peak bone mass occurs soon after puberty, lasting until 40-45 years, an age after which age-dependent bone loss begins [1]. We also suggest, that puberty period may not affect OPG concentration. It is in accordance with Buzi et al. [21], who found no differences in levels of this marker between normal children and children with early and precocious puberty.

The relationship between serum concentrations of endogenous OPG and bone turnover are still unclear, with different studies yielding different results [22,23]. Some authors suggest that OPG in adults is associated with a profile of bone turnover markers favouring bone formation [24]. Therefore this marker may be protective factor against bone resorption and age-related bone loss.

In conclusion we report an age-related decrease in circulating endogenous OPG in children and adolescents. Serum OPG concentration in postpubertal period may be similar to those presented in young adults. Prospective studies are needed to investigate the influence of OPG on bone metabolism in children.

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Event-related cerebral potentials for the diagnosis of subclinical hepatic encephalopathy in patients with liver cirrhosis

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Abstract

Purpose: Subclinical hepatic encephalopathy (SHE) seems to be a common problem in liver cirrhosis, however, studies assessing the pathogenesis of this disease remain unclear.

Currently no gold standard exists for the diagnosis of this complex neuropsychiatric syndrome. The present study was undertaken firstly to examine the diagnostic usefulness of auditory event-related cerebral potentials (ERPs) in the detection of SHE, and secondly to compare it with that of the most validated psychometric test.

Material and methods: 22 patients with liver cirrhosis without overt hepatic encephalopathy and 28 healthy controls were studied, using auditory ERPs. In addition they underwent a battery of neuropsychological and laboratory tests.

Results: P300 latency analysis turned out that cirrhotics patients had significantly longer P300 latency than controls. The only neuropsychological test showing significant difference between clinical and control group was the similarities subtest of WAIS-R.

Conclusions: The results of the present study suggest that ERPs are more sensitive method than psychometric tests in detecting early changes in the brain function of patients with cirrhosis and for this reason this neurophysiological method should be applied in clinical practice.

Key words: subclinical hepatic encephalopathy, neuropsychological tests, event-related cerebral potentials (ERPs).

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Introduction

Subclinical hepatic encephalopathy (SHE) has been definied as a condition in which patient with cirrhosis, regardless of its etiology, demonstrate a number of quantifiable neuropsychological defects, yet, on clinical examination have a normal mental and neurogical status [1]. The prevalence of SHE has been reported to vary from 30% to 84%, depending on the tests and population used [2]. Recently, several investigators have used neurophysiological tools such as quantitative electroencephalogram (EEG) analysis and exogenous or endogenous evoked potentials (EP) for the diagnosis of SHE [3]. The use of exogenous EP failed to detect SHE with an accurate sensitivity. However, the use of endogenous event-related cerebral potentials (ERPs) seems to be really helpful in diagnosis of SHE [4,5].

The P300 complex of visual or auditory event-related potentials appears to be frequently and consistently abnormal in cirrhotic patients with subclinical encephalopathy, in contrast to early components N100 and P200 which do not differentiate between SHE group and controls [3]. The P300 latency, which is reported to reflect the duration of evaluation process for an event or a stimulus, is significantly prolonged in cirrhotic patients than in controls. In the recent auditory ERPs study of Saxena et al. the mean P300 latency came to 363.5 msec in cirrhotic patients with SHE versus 349.3 msec in controls [6]. In addition, the mean P300 latency of cirrhotic patients, who were more than 40 years of age were significantly prolonged (369.9 msec) when compared with the latency of the younger cirrhotic patients (351.9 msec). Similarly, in the visual ERPs study of Giger-Mateeva et al. P300 latency of some cirrhotic patients without overt encephalopathy significantly exceeded the corresponding mean for controls [7]. Jones et al. have found in the visual ERPs study that the prolongation of P300 latency in cirrhotic patients without overt HE may occur in the absence of abnormalities of a standard psychometric test [8]. This neurophysiological finding discloses the existence of a delay of the stimulus evaluation time in cirrhosis even without

overt hepatic encephalopathy. Some investigators suppose that the P300 latency prolongation can originate from structural abnormalities of astrocytes known from Alzheimer's disease [9]. Others suggest that these abnormalities reflect metabolic neuropathophysiological, rather than structural, neuronal changes. The reversibility of at least some prolonged latencies of P300 is consistent with this concept [8,10].

However, controversy exists in the literature whether this neurophysiological method is as sensitive as psychometric tests. In the recent auditory ERPs study of Senzolo et al. P300 analysis did not prove very useful in discriminating patients with SHE [11]. Similarly, in the auditory ERPs study of Amodio et al., P300 latency provided little additional information for diagnosis of subclinical hepatic encephalopathy [12]. The present study was therefore undertaken firstly to examine the diagnostic usefulness of auditory P300 in the detection of SHE, and secondly to compare it with that of the most validated psychometric test.

Material and methods

For the experiment, 22 patients with a diagnosis of chronic cirrhosis were selected (11 women and 11 men). They all had no symptoms of focal damage to the central and peripheral nervous system and had no subjective feeling of lack of any deficits in the nervous system.

They were undergoing treatment in the University Hospital of Jagiellonian University in Cracow. The aetiology of cirrhosis was based on: clinical features, laboratory tests (blood plasma measures of: AST, ALT, albumines, prothrombin time, bilirubin, ammonia), serological tests of HBsAG, anti HCV antibodies, autoimmune markers as appropriate, history of alcohol and drugs abuse, genetic tests for haemochromatosis, ultrasonography to measure liver span, echo parenchyma and portal vein diameter, to confirm portal hypertension and ascites, endoscopy to check for presence of esophageal varices, where possible (no coagulopathy) liver biopsy for histopathological assessment.

Exclusion criteria from the clinical group were encephalopathy symptoms or signs of pathology observed in neurological assessment. Ages in the clinical group ranged from 27 to 59 (mean=48.7 years, SD=10.5). All the subjects were volunteers and were asked to participate in the experiment by the doctor taking care of them (participation was not enforced in any way and about 2/3 of patients agreed to participate).

Beside the clinical group 28 healthy subjects (20 women and 8 men) were included into the study as the controls. Their age was matched to the clinical group (mean=45.7; SD=10.9). All of them reported being free of neurological and psychiatric disorders.

All the subjects passed a battery of neuropsychological tests to assess for any cognitive deficits.

Nuclear Magnetic Resonance

All subjects were examined in TSE T1, TSE T2 and FLAIR sequence in transverse and sagittal planes with slices 3 mm and 5 mm thick. It revealed no focal damage to the CNS.

Neuropsychological Tests

To assess for any cognitive deficits, a battery of neuropsychological tests was used. They included (in order of applying): tapping test – right hand, tapping test – left hand (mean of 5 trials), memory test – spontaneous recall of 2 logical texts (8 and 16 positive elements) with recall of the first text afterwards (distraction), one-minute trials of verbal fluency: 2 semantic categories (animals and plants), 2 phonological categories (Polish nouns beginning with 'k' and 's' – chosen because of the highest noun frequency in Polish), Colour and Shape Sorting Test (the result of 2 categories found was treated as correct), Trail Making Test A+B (time of execution was analyzed), WAIS-R (Polish version), Beck Depression Inventory (score below 12 was interpreted as lack of depression symptoms). All the tests were run in a single session, which lasted approximately 2 hours.

Results of all tests and statistical significance of the difference between control and clinical group are depicted in *Tab. 1*. The only test showing significant difference between clinical and control group was the similarities subtest of WAIS-R test.

EEG recording and analysis

Scalp voltages were collected from Ag/AgCl active electrodes (with pre-amplifiers) using the 32-channel BioSemi Active-Two system. Electrodes were secured in an elastic cap (Electro Cap International). The vertex electrode was used as reference. Afterwards, after the recording, the data were re-referenced to linked electrodes FT7, T7, TP7, FT8, T8, TP8. The vertical electrooculogram (EOG) was recorded from electrodes placed above and below the right eye. The horizontal EOG was recorded from electrodes positioned at the outer canthus of each eye. The electrical signals were digitized within a sampling rate of 512 Hz. A digital bandpass filter set from 0.1 to 40 Hz (attenuation – 24 dB) was applied to all the data prior to running analyses, in order to reduce high frequency content, irrelevant to the components of interest. Additionally notch filter (50 Hz) was applied to the data.

Procedure

The experimental session took place in a dimly-lit sound-attenuated chamber. The participants were seated in a comfortable chair and presented a series of auditory stimuli. They all followed the odd-ball paradigm [13]. Two types of auditory stimuli were presented to them: "standards" (2 kHz, 85% chance of appearance), and "deviants" (1 kHz, 15% chance of appearance) in a random order. Each tone lasted for 100 milliseconds (rising/falling time 10 ms), and was presented at the level of 60 dB. The interstimulus interval was random, within the range of 900-1100 milliseconds.

The experiment was run in two phases – "passive" and "active". During the passive phase subjects were instructed just to listen to the sounds and do nothing. During the "active" phase subjects were instructed to listen to the sounds and count the rare tones (the 15% minority of 1 kHz tones). Additionally subjects were instructed to try not to fall asleep and keep their eyes open, try to refrain from blinking and shaking their heads (in order to minimize electric artifacts).

Both parts of the experiment lasted until 80 (+/-5) deviant stimuli were presented (which usually lasted 10 minutes).

Table 1. **Comparison of results of neuropsychological tests between clinical and control group. Significant differences are written in bold letters**

	Clinical group		Control group		p	test used
	mean	SD	mean	SD		
Tapping test						
right hand	45.1	9.2	43.8	7.6	0.38	
left hand	41.4	7.3	40.5	7.4	0.48	
Trail Making Test						
part A (time)	49.5	17.5	45.3	17.5	0.34	
part B (time)	102	49	90.7	39.8	0.25	
WAIS-R (PL)						
general IQ	98.2	103.0	13.9	18.0	0.33	
verbal	105.2	109.0	13.8	17.8	0.43	
information	10.2	10.5	2.3	3.1	0.77	
digit span	8.4	8.8	2.6	3.4	0.64	
vocabulary	13.2	13.5	2.4	2.6	0.67	
arithmetic	10.2	11.3	2.6	3.0	0.19	Student-t Test
comprehension	12.2	12.2	2.7	2.7	0.98	
similarities	**9.1**	**10.9**	**2.6**	**2.8**	**0.03**	
performance	91.3	97.5	12.7	13.3	0.12	
picture completion	9.1	10.4	2.9	2.9	0.12	
picture arrangement	7.5	8.7	2.0	2.3	0.07	
block design	8.3	9.5	2.4	2.6	0.14	
digit symbol	8.1	9.2	2.8	2.5	0.16	
object assembly	8.8	9.0	2.3	2.2	0.86	
Memory test						
text I	7.3	1.0	7.3	0.9	0.95	
text II	9.6	3.6	8.7	3.6	0.42	
text I (distraction)	5.6	2.9	5.6	2.8	0.95	
	N	**%**	**N**	**%**		
Verbal fluency						
animals	23.5	7.1	22.1	6.4	0.49	
plants	21.3	6.3	19.0	6.6	0.26	
letter ‘k’	11.4	5.6	12.9	5.6	0.38	chi-square Test
letter ‘s’	12.1	4.1	11.2	4.3	0.49	
Sorting (2 categories found)	18	95	23	88	0.47	
Beck Depression Inventory (score <12)	8	42	13	48	0.69	

Between the phases subjects were given opportunity to rest for 5 minutes. The “passive” phase was included only in order to calibrate and validate the hardware, and its results are not included into this work (moreover, its results did not differentiate the two groups).

Whole procedure lasted approximately 25 minutes for both parts not including the time needed for electrodes placement.

Data analyses

Continuous EEG recording was divided within each subject into 1 second epochs, time-fixed to the onset of the target tones (deviant stimuli). Each epoch started 100 msec before the onset of the stimuli, and ended 900 msec after. After that those 1000 msec epochs were averaged into one epoch, giving so-called event-related potential.

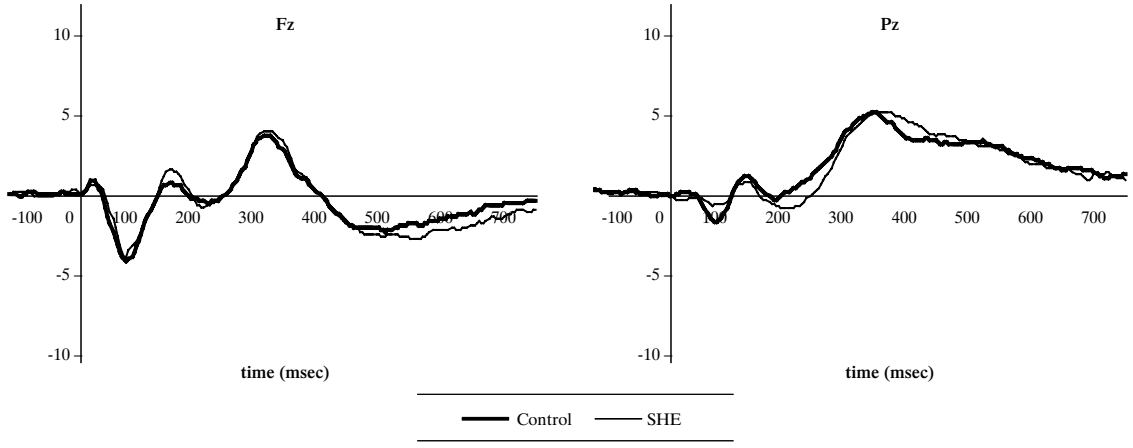
Baseline correction was performed using the average EEG activity in the 100 msec preceding the onset of the target tone as a reference signal value. Following baseline correction ocular correction was performed using Gratton and Coles algorithm [14].

After that, trials containing artifacts on electrodes of interest (Fz, Pz, and the reference electrodes) were rejected. The criteria were: amplitude beyond +/- 100 μV; change of amplitude within one trial greater than 150 μV, steepness of change greater than 35 μV per msec. Less than 15% of the trials were excluded by this operation.

The P300 component was defined as the largest positive-going peak occurring as a reaction for the deviant stimuli within a specific latency window: 250-450 msec. Peak amplitude was measured relative to the prestimulus baseline (100 ms), and peak latency was measured from the time of stimulus onset.

In addition to statistical tests, percent of abnormal P300 latency results was reported. The criterion for qualifying scores as normal or abnormal was taken from Polich et al. [15]. It defines formula for computing Z-score for P300 latency taking age under consideration ($Z = [\text{actual value}-(250+1.4*\text{age})]/40$). Values of $Z>2$ were considered abnormal.

Figure 1. **Event-related potentials for deviant stimuli in odd-ball paradigm on electrodes Fz and Pz – comparison of control and clinical groups**



Results

Fig. 1 depicts grand averages (averages of event-related potentials for all subjects within each experimental group) for the deviant stimuli from clinical and control groups for two electrodes position: Fz and Pz.

Two characteristics of the P300 component were compared – its amplitude and latency (their definition is described in Data Analyses section). Two Student's T-tests were run. For P300 amplitudes there was no significant difference between control and clinical groups (means 7.72 and 7.75 accordingly). P300 latency analysis turned out that cirrhotics had significantly longer P300 latency than controls – on average by 22 ms (395 and 373 accordingly; $t=2.35$; $p<0.05$).

Beside that, P300 was correlated with demographic and neuropsychological variables, to see if they influenced P300 latency on whole population (controls + cirrhotics). It turned out that there were two other variables significantly linearly influencing the latency: age ($r=0.39$; $p<0.01$) and the result in Beck Inventory ($r=0.41$; $p<0.01$).

To see if any of these variables explained variance of any other, P300-correlated variable, all three variables (group, age and Beck result) were entered into linear hierarchical regression analysis. Beck result was entered as the first variable, then group, and then the age. At the end only Beck's and group's betas were significant (Beck beta=0.331; group beta=0.31) and age didn't exceed significance threshold (beta=0.23). It turned out that the age had some common variance with the result of Beck inventory ($r=0.39$; $p<0.05$). Whole model of regression was significant ($R^2=0.28$; $p<0.01$).

Additionally, further correlations were looked for inside the clinical group only, between the P300 latency and the biochemical variables. It turned out that level of ALAT enzyme was significantly correlated with P300 latency ($r=-0.51$; $p<0.05$).

Beside the statistical analysis, standard count of normal/ /abnormal P300 latency was performed. It turned out that among the clinical group there were 8 subjects which qualified as abnormal (36%); in the control group there were 7 subjects with abnormal P300 latency (25%).

Discussion

The present study has demonstrated that latency of P300 component of ERPs are significantly prolonged in cirrhotic patients without overt encephalopathy compared with P300 latency in controls. Cirrhotic patients with prolonged latency of P300 may have subclinical hepatic encephalopathy. Our findings complement and extend previous reports on event-related cerebral potentials in patients with liver disease. The ERPs studies have been shown to be of great value in detecting latent stages of encephalopathy [6-8]. As opposed to conclusion of some investigators (Amodio et al., Senzolo et al.) we have demonstrated that P300 latency can provide useful information for diagnosis of cirrhotic patients in clinical practice [11,12].

An important finding of our study is that ERPs performed better than any of the neuropsychological screening tests used in detection of SHE. The event-related potentials seem to be more sensitive method than psychometric tests in detecting early changes in the brain function of patients with cirrhosis and for this reason this method should be applied in the assessment of them.

It is generally accepted that a lot of factors like age or severity of liver disease could influence the neurophysiological data and for this reason they were included in regression analysis. Results revealed that among the various factors in our model, only two had significant importance: group (patients vs controls) and depression symptoms. Many studies of brain function have suggested that depression is associated with cerebral hypoactivity. The latency of P300 component was found to be significantly prolonged in cases of major depression as compared to that of controls [16,17]. The results of our study are consistent with these findings. However, further investigations are required to

elucidate the diagnostic and predictive role of latency of P300 in the cases of depression. What is the most important, this result do not minimize the influence of liver disease on prolongation of P300 latency in our study. In addition, P300 latency prolongation has appeared as independent of aging. It means that age factor may be less important in neurophysiological diagnosis of SHE than some investigators has suggested [12].

It is only important to remember to control for all possible confounds of latency while matching patients in the control group. Age seems to be one such factor, depression level seems to be another. To our knowledge there is no study on P300 latency and SHE which controlled for depression. It is especially important when subjects are divided into normal and abnormal P300 latency groups. The rule of thumb was to qualify as abnormals subjects whose latency was 2 standard deviations off the norm. Recently what is norm for P300 latency has been adjusted for age factor [12]. It may be also important to take depression under consideration, especially that it showed stronger influence than age.

The present study has demonstrated that neurophysiological tests can add valuable information in the assessment of early stages of encephalopathy compared to the test battery and they should be used in the diagnosis of this neuropsychiatric syndrome. The auditory event-related potentials represent a promising tool for the objective diagnosis of subclinical hepatic encephalopathy for many reasons. Firstly, this method is reproducible, relatively easy to perform, non-invasive and safe to implement as compared to the other brain structural or metabolic studies. Secondly, it is more sensitive for the diagnosis of SHE than the established psychometric tests. Thirdly, it is not influenced by learning effects by the patient. We conclude that auditory ERPs are strongly recommended for diagnosis of subclinical hepatic encephalopathy.

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Influence of nutritional treatment on the postoperative course in patients with gastric cancer

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Abstract

Purpose: Malnutrition occurs in ca. 60% of all patients with gastric cancer. The obligatory standard for a curative radical oncological procedure is gastrectomy inclusive of regional lymph nodes. Nutritional treatment is expected to decrease possibilities of postoperative complications in patients subjected to curative surgery. The study is aimed at comparing treatment results in patients with gastric cancer subjected to radical surgery, nutritional and non-nutritional treatment respectively.

Material and methods: The study included 176 patients qualified for curative surgery of a total or subtotal gastrectomy. Analysed were 2 groups of patients: group I – not subjected to nutritional treatment, group II – subjected to nutritional treatment, both in the circumoperative period. The groups were compared in respect to: 1) age, 2) sex, 3) nutritional condition, 4) degree of clinical cancer development, 5) histopathological cancer type, 6) kind of surgical procedure performed, 7) antibiotic and antithrombotic prevention. All complications observed in the patients were divided into four kinds: surgical of a high or low risk and general of a high or low risk.

Results: Given the above-mentioned estimation parameters, no statistically significant differences between both groups were recorded. Of 176 patients, 27% showed surgical complications and 40% had general complications. No difference ($p=0.60$) in the incidence of a high and low risk surgical complications between groups I and II in the circumoperative period was observed, a significant difference ($p=0.03$) was recorded in the incidence of general complications. Low risk general complications (respiratory infections) were shown to occur significantly more often ($p=0.005$) in patients receiving either parenteral or enteral nutrition after surgery.

Conclusions: A significant part of the patients with a medium degree and a medium to heavy degree of malnutrition subjected to a curative gastrectomy can pass through the postoperative period without using either parenteral or enteral nutrition and with no violations of all the other principles of the postoperative procedure as well as without provoking any significant increase of surgical complications. In case surgical complications should occur and delay resuming natural feeding, it is necessary that parenteral and/or enteral nutritional treatment be undertaken according to clinical circumstances and condition of the patient concerned; such proceedings increase chances of cure.

Key words: gastric cancer, gastrectomy, nutritional treatment, complications.

Introduction

Malnutrition in patients with malignant alimentary tumours develops in 30-80% cases subject to which organ is affected. It occurs in ca. 60% cases of all gastric cancer patients [1,2]. The obligatory standard for a curative radical oncological procedure is a surgical treatment, i.e. gastrectomy inclusive of regional lymph nodes [3-6]. Nutritional condition of the patients having to be subjected to an extensive surgical procedure due to gastric cancer, seems very significant for the effective surgical treatment [7-9]. Parenteral and/or enteral nutritional treatment contributes to eliminating or decreasing nutritional deficiencies and helps recover a normal protein, carbohydrate and fat as well as hydroelectrolytic balance respectively prior to the surgical treatment, while in the postoperative period both or one may cause a quick shift from the phase of catabolism to anabolism. [10-15]. Hence the introduction of nutritional treatment in curative gastric tumour patients with a surgical record should

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decrease the likelihood of postoperative complications in the patients concerned.

Circumoperative patient nutrition has been widely discussed for many years. The authorities on the issue are unanimous that malnutrition deteriorates treatment results, extends in-hospital stays and increases treatment costs. Nutritional treatment is recommended in severely malnourished patients. The majority of European societies, inclusive of the Polish Parenteral and Enteral Nutrition Society, accept the opinion that circumoperative nutritional treatment is not advisable in patients showing either a proper nutritional condition or mild malnutrition who within a week following surgery are expected to resume normal feeding fulfilling 60% of nutritional demand [16]. Total or subtotal gastrectomy hinders efficient oral feeding that fulfils 60% of nutritional demand within 7 days following surgery. However, orally fed and non-orally fed patients show a similar number of septic complications following gastric cancer surgery. Given the above-mentioned, tests are conducted to replace parenteral nutrition with a manufactured diet by enteral administration or intravenous crystal liquids, electrolytes and 5% glucose solutions [17,18]. The patients scheduled for enteral treatment due to surgery in the upper digestive segment are recommended to receive a thin probe through the nose down to the small intestine, 10-15 cm below the lowest anastomosis [16]. Actually, gastric cancer patients after surgery are, in most cases, given a combined parenteral/enteral nutrition, decreasing the intravenous supply in parallel with an increasing tolerance to enteral feeding.

Of importance in selecting a proper method of postoperative procedure may be assessment of the number and kind of postoperative complications in patients with a similar level of malnutrition, subjected to curative surgery due to gastric cancer contingent on the way of postoperative course.

The study is aimed at comparing early treatment results in patients with gastric cancer subjected to curative radical surgery who were and were not nutritionally treated.

Material and methods

The study included 176 patients (58 female and 118 male) qualified for the curative surgery of a total or subtotal gastrectomy; the patients were selected from 311 of those treated due to gastric cancer in the years 1988-2003.

Detailed analysis was performed in two groups of patients:

– group I – non-nutritionally treated in the circumoperative period,

– group II – nutritionally treated in the circumoperative period.

Group I included 51 patients (15 female and 36 male) in whom nutritional treatment was impossible in the circumoperative period due to lack of patient consent, a poor tolerance to nutritional treatment trials, lack of qualification for nutritional treatment within the first years of the period concerned. The patients, who had not been nutritionally treated, received an oral diet in the preoperative period, and were postoperatively given adequate rations of liquids and electrolytes inclusive of ca 300 kcal per day in the form of 1.5 litre of 5% glucose. In Group II (125 patients – 43 female, 82 male) was introduced nutritional treatment in the form of parenteral nutrition (PN), enteral nutrition (EN) or combined parenteral/enteral nutrition. Oral supply of liquids was introduced in the 5th day following surgery, control of the anastomosis tightness was performed on the 7th day by means of uropoline. If the anastomosis proved tight, oral feeding was decided and hospital diet given.

An average age of non-nourished patients was 62.3, and nourished ones 62 years.

For the sake of comparison the following criteria were analysed in both groups: 1) age, 2) sex, 3) nutritional condition, 4) clinical staging of cancer according to the TNM UICC classification of 1997 [4,6], 5) histopathological cancer type according to the Lauren classification [4,6], 6) kind of surgical procedure performed, 7) antibiotic and antithrombotic prevention introduced.

Nutritional condition of the patients was assessed on admission to hospital with respect to:

– subjective, global assessment of nutritional condition – SGA (Subjective Global Assessment) [2],

– anthropometric examinations – percentage of body mass loss in the most recent 3 months prior to treatment commence, BMI [2],

– laboratory examinations – concentration of albumin in the blood serum, total lymphocyte count, concentration of total protein in the blood serum.

The patients subjected to thorough SGA-based assessment of nutritional condition were qualified to the following groups representing: 1) proper nutritional condition, 2) mild malnutrition, 3) medium malnutrition, 4) severe malnutrition. Chosen anthropometric examinations based on the height and body mass of the patient helped to determine a body mass index (Body Mass Index – BMI), and to define the disease-related loss of body mass within the period of 3 months prior to treatment commence [2]. Results of laboratory examinations were assessed before surgery, i.e. on admission to hospital. The minimum level of albumin concentration, total lymphocyte count (TLC) and concentration of total protein were agreed at the following amounts respectively: 35g/l, 1500 in mm^3, 63 g/l. BMI values between 18 kg/m^2 and 20 kg/m^2 as well as body mass loss above 5% within the period of 3 months prior to admission to hospital were found to prove the risk of malnutrition and qualifying for nutritional treatment. Severe malnutrition was indicated by the BMI below 18 kg/m^2, body mass loss above 10% within the period of 3 months prior to treatment commence, TLC < 800 in mm^3.

To assess the degree of gastric cancer development, the following were performed in 176 patients:

– clinical examination,

– endoscopy with taking specimens for histopathological examination,

– radiological examination of the chest,

– ultrasound and CT of the abdominal cavity,

– exploration of the abdominal cavity organs by the operator,

– macroscopic and microscopic examination of the operative preparation [4,6].

In the premedication period (30-45 minutes before surgi-

cal procedure) the patients were administered an intravenous antibiotic prevention (Cephalosporine I + metronidazol) and a subcutaneous antithrombotic prevention (low-molecular-weight heparin). A kind of surgical procedure was chosen based on surgical treatment standard guidelines in an early and advanced gastric cancer. Performing a subtotal excision of gastric cancer, 4/5 part of the stomach was resected, a margin was left from the edge of the tumour with Lauren I – 5 cm and Lauren II – 7 cm towards the incision line [4,6]. The digestive tract continuity reconstruction was performed by means of the following techniques: Billroth I, Billroth II, "omega" with the Braun anastomosis and Roux – en – Y in accordance with to the standards obligatory in these techniques.

All complications observed in patients were divided into 4 categories:

– surgical of a high risk – dehiscence of esophagointestinal anastomosis, dehiscence of gastrointestinal anastomosis, dehiscence of duodenal stump, bleeding from the upper segment of the digestive tract;

– surgical of a low risk – surgical wound suppuration, peritoneal fluid collection, suppuration in the canal after removing the drainage tube, an IV cannula reaction;

– general of a high risk – cardial infarct, brain stroke, respiratory insufficiency, pancreatitis, circulatory insufficiency, kidney insufficiency;

– general of a low risk – fevers without no observable cause, pneumonia and bronchitis, pleural effusion, diarrhoea, urinary infections.

Statistical analysis was performed to find a dependence between monitored parameters and the incidence of surgical/ /general complications in both groups of patients. In order to verify hypotheses concerning lack of dependence between "quality" features discussed and complications, the chi^2 independence test was used. In order to verify hypotheses concerning lack of crucial differences between median values of "quantity" features in the groups of patients with a diverse degree of postoperative complication risk, the single-factor variance analysis based on the F test was used. A p values <0.05 were considered statistically significant.

Results

Examined were 176 patients with curative gastric cancer treated by means of resection surgery. Group I included 51 (29%) patients in whom nutritional treatment was not introduced, whereas group II consisting of 125 (71%) patients were nutritionally treated. A comparative analysis was made in both groups of the patients.

On closer analysis of age distribution in the groups in respect to sex, no statistically significant difference in the number of women and men either in group I ($p=0.08$) or in group II ($p=0.13$) was found.

Thorough SGA-based assessment of nutritional condition showed that on admission to hospital the patients were medium or severely malnourished in most cases: in group I – 94%; in group II – 95%. The BMI analysis showed that 24% of both non-nourished and nourished patients proved qualified for nutritional intervention (BMI <20 kg/m^2). No statistically significant difference ($p=0.39$) of BMI values between group I and II was recorded. Loss of body mass indicating medium malnutrition occurred in 18% of the patients from group I and 38% of the ones from group II. Severe malnutrition was observed in 70% and 56% of the patients respectively. Albumin concentration in the blood serum below the norm was found in 41% of non-nourished patients and 39% of nourished ones. Total lymphocyte count below 1500/mm^3 was found in 66% of the patients from group I and 41% from group II. Total protein concentration below the accepted norm was observed in 42% of non-nourished patients and in 36% of nourished ones. No statistically significant difference was found between both of the groups with respect to body mass loss ($p=0.37$), albumin concentration ($p=0.10$), total lymphocyte count ($p=0.40$), total protein concentration ($p=0.65$). Both of the analysed groups showed that the patients had an advanced cancer in a majority of cases, mostly tumours T3: 70% in group I; 54% in group II. Nodal metastases were observed in 55% of the patients in group I and 66% in group II; distant metastases 8% and 10% respectively. Group I included most of the patients having an intestinal cancer (49%), while group II a spread type of cancer (50%). No statistically significant difference in the size of the tumour ($p=0.11$) was recorded as regards lymph metastasis incidence ($p=0.55$) and distant ones ($p=0.65$), histopathological cancer type ($p=0.18$) observed between non-nourished patients and nourished ones in the circumoperative period. Group II included in a statistically significant way ($p=0.01$) more frequent cases of a total gastrectomy than a subtotal gastrectomy.

Of 176 patients operated due to gastric cancer, complications affected 91 patients (52%). Circumoperative mortality amounted to 5.1%. Surgical complications occurred in 47 (27%) patients, general complications in 70 (40%).

Low risk surgical complications included 5 (9.8%) patients in the non-nutritionally treated group whereas in the nutritionally treated group 18 (14.4%) patients. High risk surgical complications are respectively as follows: group I 6 (11.8%), group II 18 (14.4%). No statistically significant difference ($p=0.60$) in low and high risk surgical complication incidence between the non-nourished and nourished patients in the circumoperative period was noted.

Low risk general complications were recorded as follows: in group I in 8 (15.7%), in group II in 45 (36%); high risk general complications in I – 6 (11.8%), in II – 11(8.8%). Statistically significant difference ($p=0.03$) was recorded in general complication incidence between non-nourished patients and nourished ones in the circumoperative period. Low risk general complications occurred above two times more frequently in the nutritionally treated patients, the highest number of them included respiratory-related infectional complications. High risk general complications occurred with the same frequency in both of the analysed groups.

Postoperative parenteral nutrition was introduced in 119 patients with 57 ones excluded. Low risk general complications occurred in 8 (14% – 8/57) non-PN-treated patients following surgery and in 45 (37.8% – 45/119) PN-treated patients following surgery. 53 patients were enterally nourished, 49 of them also received parenteral nutrition, of 176 examined patients,

123 were not enterally nourished. Low risk general complications occurred in 28 (22.8% – 28/123) non-EN patients and in 25 (47.2% – 25/53) patients after EN. Low risk general complications occurred significantly more frequently ($p=0.005$) in the patients receiving the postsurgical PN and EN. No differences in surgical and general complication incidence was observed either in the circumoperatively non-nutritionally treated patients or nutritionally treated patients after a total and a subtotal gastrectomy.

Suppurative complications (surgical wound suppuration, peritoneal fluid collection, suppuration in the canal after removing the drainage tube) that were included in low risk surgical complications, occurred in 16 cases of 125 (12.8%) nourished patients and 5 cases of 51 (9.8%) non-nourished patients. Moreover, in the nutritionally treated patients was observed 6/125 (4.8%) post-IV cannula reactions which were not recorded in the group of non-nutritionally treated patients at all. Tube-related reactions should be the reason of a higher number of low risk surgical complications in nourished patients in the circumoperative period as compared with the patients malnourished. Anastomosis dehiscence is the most serious high risk surgical complication. The very complication occurred as the only high risk surgical complication in 6 malnourished patients – 6/51 (11.8%). In the nutritionally treated group of the patients in the circumoperative period, anastomosis dehiscence occurred in 16 patients – 16/125 (12.8%) as well as there were 2 cases of bleeding from the upper segment of the digestive tract. The anastomosis dehiscence occurred with a comparable frequency both in the group of non-nutritionally treated patients and nutritionally treated patients in the circumoperative period.

Among low risk general complications in nutritionally treated patients, a majority included respiratory infections. Pneumonia and bronchitis, pleural effusion occurred in 29 cases of all 125 (23.2%) nutritionally treated patients in the circumoperative period. Fever with no evident cause occurred in 26 cases of all 125 (20.8%) patients in that group. In the group of malnourished patients, the following were recorded respectively: 3/51 (5.9%) pulmonary infections and 5/51 (9.8%) fever showing a four-times reduction in pulmonary infections and a two-times reduction in the fever incidence. High risk general complications in both of the analysed groups were mainly circulatory-related disturbances. Circulatory insufficiency, cardial infarct and brain stroke occurred in 6 cases of 51 non-nutritionally treated patients (11.8%) as well as in 5 cases of 125 nutritionally treated patients (4%). Respiratory insufficiency was not observed in group I patients, whereas 4 such cases of 125 (3.2%) patients were recorded in group II.

Discussion

Decrease in the number of surgical complications, particularly those of a high risk is envisaged by applying the circumoperative nutritional treatment in malnourished patients. In the study presented, the number of surgical complications in nutritionally treated patients is not lower than in non-nutritionally treated ones. Explanation was sought through a detailed analysis of which of the complications had occurred in the patients.

The presented study includes 176 gastric cancer patients divided into group I, i.e. non-nutritionally treated and group II i.e. nutritionally treated in the circumoperative period. No significant difference in the nutritional condition between both groups prior to surgery was found. Despite lack of nutritional intervention, group I patients did not show a complication incidence higher than in group II patients. Lack of nutritional treatment in the patients did not contribute to any increase in the incidence of high risk surgical and general complications. Non-surgical infectional complications, i.e. low risk general complications occurred in nutritionally treated patients more frequently than in non-nutritionally treated ones. It serves to confirm the assumption raised by the authors of some publications that nutritional treatment following surgical procedures increases the number of infectional complications in the patients subjected to this kind of therapy with no explainable cause. Bellantone et al. [19] found a two-times increase in the incidence of infectional complications in parenterally nourished patients, if they were not found to be severely malnourished in the preoperative period. The performed examinations have shown that it is possible to pass a gastric cancer patient safely through the circumoperative period without nutritional treatment, but still with keeping to all the other rules of surgical procedure.

Komorowski et al. [18] have stated that a routine use of parenteral nutrition in the postoperative period is not justified in patients showing no serious nutritional deficiencies. Nutritional treatment is recommended in severely malnourished patients in whom it may decrease surgical complication risk. The studies by Veterans Affaires [20] and Bozetti et al. [17] have shown that in severely malnourished patients subjected to surgical procedures due to digestive cancer, parenteral nutrition reduces the incidence of non-infectional complications resulting in the incidence of non-surgical infectional complications – particularly during use of parenteral nutrition. Braga [21,22], Pawłowski [23], Papapietro [24], Sand [25], Grahm [26], Hoyer [27] and Wells [28] have shown in their studies that enteral nutrition is well tolerated and should be the treatment of choice in the patients subjected to gastrectomy due to cancer. Therefore, from the moment a gastric cancer is suspected, the nutritional procedure should include a natural diet supplemented with manufactured diets, and a combined parenteral/enteral nutrition should be introduced after surgery, with decreasing the intravenous supply in parallel with an increasing tolerance to enteral feeding.

Conclusions

A significant part of the patients subjected to a curative gastrectomy with a medium degree of malnutrition and a medium to heavy degree of malnutrition may pass through the postoperative period without using either parenteral or enteral nutrition, and still keeping to all the other rules of the postoperative procedure and without provoking any significant increase of surgical complications.

In case surgical complications should occur and delay resuming natural feeding, it is necessary that parenteral and/or enteral nutritional treatment be undertaken according to clini-

cal circumstances and condition of the patient concerned; such proceedings increase chances of cure.

In gastric cancer patients with a medium degree of malnutrition and a medium to heavy degree of malnutrition, who were non-nutritionally and nutritionally treated after curative resection procedures, the incidence of surgical and general complications is similar.

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Anaplasma phagocytophilum in *Ixodes ricinus* ticks and human granulocytic anaplasmosis seroprevalence among forestry rangers in Białystok region

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Abstract

Purpose: Human granulocytic anaplasmosis, former ehrlichiosis, is a tick-borne zoonosis of increasing recognition.

The aim of the study was: 1) to assess the prevalence of *Anaplasma phagocytophilum* infection in *Ixodes ricinus* ticks collected in recreational forests in Białystok vicinity, the capital of podlaskie voivodship; 2) to evaluate the prevalence of IgG and IgM antibodies to *A. phagocytophilum* among forestry rangers from the same region.

Results: Of the 372 ticks examined, 54 (14.5%) yield the positive PCR reaction. The highest prevalence was detected in females, up to 27.8% (37/133), almost one third lower in males – 9.2% (13/142), followed by nymphs – 4.1% (4/97). Human seropositivity study revealed IgG antibodies against *A. phagocytophilum* in 9 out of 231 individuals (3.9%). No IgM antibodies were found. Sixty-seven individuals 67/231 (29%) reported erythema migrans. IgM anti-*Borrelia burgdorferi* antibodies were detected in 32 out of 121 (26.4%) persons tested, IgG – in 43 out of 231 (18.6%).

Conclusions: The data obtained show relatively low *A. phagocytophilum* seroreactivity among professionally exposed to tick group of forestry workers despite high *A. phagocytophilum* infection level in the competent vector – *I. ricinus* ticks.

Key words: *Anaplasma phagocytophilum*, *Ixodes ricinus*, ticks, antibodies, human granulocytic anaplasmosis, forestry rangers, Białystok region, Poland.

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Introduction

Anaplasma phagocytophilum is a unique bacterium infecting and multiplying successfully in granulocytes of broad range of hosts, including domestic and wild animals – canids, horses, sheep, cattle, European bisons and rodents, as well as humans. Infection may be subclinical or manifesting as a non specific febrile disease, called granulocytic anaplasmosis, tick fever or pasture fever in sheep [1-3]. *A. phagocytophilum* is transmitted by ticks from *Ixodes persulcatus* complex, which in Europe is mainly *I. ricinus* [1,4]. Ticks and tick-borne diseases are endemic in north-eastern Poland. This region has also the highest incidence of tick-borne encephalitis in Poland – 7.8/100 000 vs 0.46/100 000 for the whole country in 2005 and Lyme borreliosis (63.1 vs 11.5) [5]. Ecological and socio-economical changes in our region lead to increased ticks abundance and therefore augmented exposure of human population to tick transmitted pathogens.

Forestry workers constitute the professional group greatly exposed to ticks. The aim of the study was: 1) to assess the prevalence of *A. phagocytophilum* infection in *I. ricinus* ticks collected in recreational forests in Białystok vicinity, the capital of podlaskie voivodship; 2) to evaluate the prevalence of IgG and IgM antibodies to *A. phagocytophilum* among forestry rangers from the same region.

Material and methods

Ticks

Host seeking ticks were collected by flagging lower vegetation in different forested areas in Białystok vicinity – Pietrasze, Strzelnica, Dzikie and in Knyszyn Primeval Forest – Bobrowa, Korytne, Supraśl-Pólko, Królowy Most and in Biebrza National Reserve, *Tab. 1*. Collected ticks were individually evaluated prior to DNA extraction by a qualified entomologist with regard to species and gender according to Siuda [6]. The ticks were killed in hot water, placed in separate vials (adult) or pooled by

Table 1. Anaplasma phagocytophilum **infection rate in host seeking** *Ixodes ricinus* **ticks collected in different forested areas in north-eastern Poland**

Collection site	Year	No positive *I. ricinus*/No tested (% positive)				
		Female	Male	Total adults	Nymphs	Total
Białystok – Pietrasze	2003	6/36 (16.6)	4/49 (8.2)	10/85 (11.8)	-	10/85 (11.8)
Białystok – Strzelnica	2001	1/3 nc	0/4	1/7 nc	-	1/7 nc
Supraśl – Pólko	2006	8/28 (28.5)	5/24 (20.8)	13/52 (25.0)	1/31 (3.2)	14/83 (16.8)
Królowy Most	2006	12/32 (40.0)*	3/27(11.1)*	15/59 (25.4)#	1/23 (4.3)#	16/82 (19.5)
Bobrowa	2001	2/7 nc	0/4 nc	2/11 (18.2)	0/2 nc	2/13 (15.4)
Korytne	2001	5/21 (23.8)	1/28 (3.6)	6/49 (12.2)	2/24 (8.3)	8/73 (11.0)
Dzikie	2001	3/6 nc	0/5 nc	3/11 (27.3)	0/11	3/22 (13.6)
Biebrzański PN	2002	-	0/1 nc	0/1 nc	0/6 nc	0/7 nc
Total		37/133 (27.8)*	13/142 (9.2)*	50/275 (18.1)#	4/97 (4.1)#	54/372 (14.5)

nc – not calculated, No < 10; * – difference statistically significant between females and males; # – difference statistically significant between adults and nymphs

2-5 (nymphs) and fixed in 70% ethanol for further investigation by PCR for the presence of *A. phagocytophilum*.

Human study population

Study participants were recruited from the following forest inspectorates localized in Białystok vicinity: Dojlidy, Supraśl, Czarna Białostocka, Waliły in July, August 2004 or 2005. Serum samples were collected from 231 forestry workers, 40 females and 191 males, aged 49 ± 12 years. The questionnaire regarding age, length and character of employment in the forest, Lyme borreliosis and tick-borne encephalitis history, ticks exposure and actual complains such as fever, arthralgia, mialgia was filled by a physician.

DNA extraction

DNA was extracted by the ammonium hydroxide lysis (NH_4OH) according to Rijpkema et al. [7]. Lysates were stored in -20°C until examination.

Polymerase Chain Reaction

PCR was performed according to Pancholi et al. [8]. The primers EHR 521 (5'-TGT AGG CGG TTC GGT AAG TTA AAG-3') and EHR 747 (5'-GCA CTC ATC GTT TAC AGC GTG-3') were used to amplify the 16S rRNA (rrs) gene fragment specific for *A. phagocytophilum*. The tick lysates from positive reactions obtained in our previous investigation [9] served as a positive control and the double distilled water as a negative control in each PCR run. All PCR reactions were carried out in Perkin Elmer GeneAmp PCR System 9700 thermal cyclers. Amplification products were analyzed after electrophoresis in a 2% agarose gel stained with ethidium bromide. DNA bands of 247 base pairs (bp) were considered positive results. All positive samples were confirmed with nested PCR reaction amplifying rrs gene according to Massung [10].

Serological tests

Human granulocytic anaplasmosis (HGA) Anti-*A. phagocytophilum* IgM and IgG antibodies were detected by indirect immunofluorescence technique applying commercial kits with HL60 cells infected with the human isolate of *A. phagocytophilum* (Focus technologies HGE IFA IgG /IgM Test Kit, USA). The serum screening dilution was 1:64 according to the manufacturer and results ≥1:64 were considered positive.

Lyme borreliosis In order to examine the anti-*Borrelia burgdorferi* serological response Borrelia recombinant IgG and/ /or IgM kits (Biomedica, Austria) applying MiniBoss were used, according to the producer.

Statistics

Statistical analysis was performed with Pearson's χ^2 test and $p \leq 0.05$ was considered significant.

Results

Ticks

Of the 372 ticks examined, 54 (14.5%) yield the positive PCR reaction. The highest prevalence was detected in females, up to 27.8% (37/133), almost one third lower in males – 9.2% (13/142), followed by nymphs – 4.1% (4/97). The overall infection rate varied depending on the collection site; among adults from 27.3% in Dzikie to 11.8% in Białystok-Pietrasze; differences in nymphs were less pronounced – from 0% to 8.3%.

Human seropositivity

IgG antibodies against *A. phagocytophilum* were detected in 9 out of 231 individuals (3.9%), *Tab. 2. A. phagocytophilum* antibodies were detected in employee from 3 forest inspectorates – Supraśl 2/36 (5.5%), Dojlidy 3/39 (7.7%) and Czarna Białostocka 4/72 (5.6%). No seropositive individuals were found neither in Żednia 0/30 nor in Waliły 0/45 inspectorates. Such a low prevalence of specific antibodies might not allow finding any significant differences depending on sex, age, length and character of forestry employment – field or office work, Lyme borreliosis serological status and history (erythema migrans), and actually reported complains (fever, arthralgia, mialgia). Surprisingly, no significant differences were detected between individuals reporting tick bites in the last year – 6/147 (2.6%) and those denying it – 3/63 (1.3%). Thus regression analysis was

***Table 2*. Prevalence of IgG against *A. phagocytophilum* and IgG/IgM against *B. burgdorferi* among forestry workers in north-eastern Poland**

Anti-*Anaplasma phagocytophilum* IgG		*Borrelia burgdorferi*							
		Erythema migrans (No=231)		IgM (No=121)			IgG (No=231)		
		+	-	+	-	+/-	+	-	+/-
Positive	9	2	7	0	4	0	0	8	1
Negative	222	65	157	32	79	6	43	170	9
Total	231	67	164	32	83	6	43	178	10

not possible. IgM antibodies against *A. phagocytophilum* were not detected in any person evaluated.

Lyme borreliosis

Sixty-seven individuals 67/231 (29%) reported erythema migrans in their medical history. Twelve foresters had erythema migrans in the year of examination, but only 3 out of 10 persons tested had IgM antibodies to *B. burgdorferi*. IgM anti-*Borrelia burgdorferi* antibodies were detected in 32 out of 121 (26.4%) persons tested, IgG – in 43 out of 231 (18.6%), however, the results were not confirmed by Western Blot tests, *Tab. 2*.

Discussion

Our results show pretty high *A. phagocytophilum* infection rate in *I. ricinus* ticks collected in north-eastern Poland, reaching 18.1% average in adults and even 40% among females in certain locations (Królowy Most). The frequency of infection significantly raises from nymphal to adult stage of ticks. This observation points to the role of little rodents and small, and intermediate mammals, harboring *I. ricinus* larvae, in *A. phagocytophilum* circulation in nature since this bacterium is not transovarially transmitted [1,4]. *A. phagocytophilum* infection rate demonstrated in our study is similar to that found in mid-eastern Poland [11]. Slovac research conducted in suburban forest near Košice revealed 12.5% adult ticks infected with *A. phagocytophilum* [12]. Several other Polish studies showed the *A. phagocytophilum* infection rates ranging from 0% in Szczecin province (Głębokie Public Bath and Landscape Park in Ińsko) [13], 13.1% in Lublin province [11], 14% in Tricity Forest on the Baltic coast [14] and to 16% in Białowieża Primeval Forest [15].

Despite the high *A. phagocytophilum* infection rate in *I. ricinus* ticks, a very low presence of antibodies (3.9%) was revealed in the present study. Beginning the investigation among forestry workers and choosing the summer months, which followed the highest tick activity, higher prevalence and eventually acute granulocytic anaplasmosis cases detection was expected. Other Polish studies in forestry rangers demonstrated higher levels of seropositivity from 17.7% (20/113) to 20.0% (13/63) in mid-eastern Poland and 9.6% (46/478) in northern and north-eastern Poland [11,16,17]. Analogically to our results, very low *A. phagocytophilum* seropositivity – 1.5% was detected in English farmers [18], nevertheless south-western German data from Baden-Württemberg forestry workers show seroprevalence ranging from 5% to 16% in various counties [19]. Northern Italy investigation showed 8.8% (16/181) sera positive by IFA, although the authors considered only one of them – 0.6%, truly positive since confirmed by Western blot [20]. However, application of Western blot is not required for granulocytic anaplasmosis diagnosis according to ESCMID Study Group [21]. All three above studies demonstrated anti-*B. burgdorferi* seropositivity surpassing those of *A. phagocytophilum* from 3 to 20 times [11, 19,20], however, the anti-*B. burgdorferi* assays results should be interpreted with caution since they where not followed by Western blot, what is required for Lyme borreliosis diagnostics [21]. The reasons of such low levels of *A. phagocytophilum* seropositivity are unclear. One of the factors may be very variable year to year tick infection rate observed in our region during 4 years period [22]. Another factor, postulated by Massung and co-workers in USA, is presence of *A. phagocytophilum* variants non-pathogenic for humans [23], however, the latter hypothesis require closer characterization of strains postulated.

The results obtained show relatively high *A. phagocytophilum* infection rate in *I. ricinus* ticks and very low seropositivity among forestry rangers, a professional group highly exposed to ticks.

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Body posture in women after mastectomy and its changes as a result of rehabilitation

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Abstract

Purpose: The aim of the study is: 1) to analyse selected features of body posture in women after mastectomy, 2) to compare them with body posture of healthy women, 3) to determine the effect of rehabilitation physical exercises on the changes in body posture in women after mastectomy.

Material and methods: The research material consisted of 85 women after mastectomy examined once, including 40 of them who were additionally examined twice at six-monthly intervals. Moreover, a group of 20 women was isolated who regularly attended rehabilitation classes for a period of one year in question. A comparative group was a group of 85 healthy women. The examinations were performed using photogrametric assessment of body posture.

Results: Distinct adverse changes in body posture of women after mastectomy in comparison with healthy women were found, manifested mainly in asymmetry of trunk and shoulder girdle and greater forward leaning of the trunk. Significant relationship was indicated between the operation of mastectomy and the asymmetry of position of scapulas.

Conclusions: When comparing the changes in the features of body posture in the group of women who exercised regularly with other women for the period of one year it was found that a positive effect of regular rehabilitation was keeping the angle of body inclination on the same level and improvement in trunk symmetry, position of scapulas and shoulder girdle.

Key words: mastectomy, posture, rehabilitation, exercise therapy.

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Introduction

Mastectomy is an operation which causes many changes in a woman's body. Its consequence are, among other things, lymphatic oedemas, limitation of movements and strength of the upper limb of the patient, experiences in the emotional sphere, difficulties related to the postoperative scar and the results of supplementing treatment such a radiotherapy or chemotherapy. Significant complications after mastectomy are changes in body posture caused both by disorders in body static as a result of amputation and limitation of movements and soreness of the spine.

The problem of changes in body posture, as a result of mastectomy is not well known. It seldom appears in scientific literature. It is, however, an important problem, both from the point of view of medical and psychological rehabilitation. Incorrect body posture may cause other somatic anomalies. For the patients good-looks related to body posture is the basis for better well-being [1,2].

Thus physicians, psychologists and patients consider mastectomy as both physical and psychological problem. Among physical impairments special attention is paid in the literature to limitations in the shoulder joint, suggesting exercise in water and swimming as effective therapy [3]. Also Reksidler [4] recommends exercises mainly in water as successful both psychic and physical therapy. On the other hand Hahn [5] suggests ski sport as effective form of therapy, bringing back self-satisfaction and diminishing depression after mastectomy.

Another argument for the physical activity recommended to women after mastectomy is based on the research conducted in women undergoing chemotherapy. In eight weeks after the surgery marching on an exercise track followed by the measurements of physiological parameters were performed. The results showed that even if the exercises caused fatigue on the same day in comparison to the days without exercise, there was no cumulative effect [6].

Lymphatic oedema and its consequences are also a problem in women who underwent mastectomy. As Schunemann et al.

Table 1. **Characteristics of the group of women after mastectomy (group A) on the first examination, the only one for this group**

Characteristics of subjects (N=85)	$\overline{X}$	min-max	SD	v (%)
Age of subjects (years)	54.6	35-79	9.6	17.6
Operation age (years)	51.4	34-79	10.4	20.3
Time from operation (years)	3.4	0-14	4.02	117.5
Weight (kg)	68.9	49- 98	12.9	18.7
Height (cm)	161.6	150-176	5.6	3.5

Table 2. **Characteristics of the comparative group of healthy women (group B)**

Characteristics of subjects (N=85)	$\overline{X}$	min-max	SD	v (%)
Age of subjects (years)	51.5	42-60	4.3	8.4
Height (cm)	164.4	150-178	5.9	3.6

showed in their study [7] among 5 868 women after mastectomy in 1 405 lymphatic oedema was observed (assessed by the increase of two centimeters of the operated limb's circumference). Physical activity is a part of antioedematous therapy.

According to many authors as Damm [8], Schulz et al. [9] and Munstedt et al. [10] motion and positive effect on the psychic should be stressed in the therapy of women after mastectomy, teaching them how to lead active and healthy lives.

A question of trunk asymmetry in the frontal plane in women after mastectomy was the concern of Dobosz et al. [11,12]. They found a frequent asymmetry relating mainly to the position of scapulas and shoulders. However, no author has ever analysed in detail the changes in body posture caused by mastectomy, in relation to the time which elapsed after the operation.

In Poland breast reconstruction is still performed rarely. Women usually obtain external breast prostheses in the form of special underwear. The aim of using this kind of supplement of the missing tissue is, among other things, compensation of changes in trunk static caused by mastectomy. Regular use of external prosthesis is very important for the effectiveness of this method of compensation for a missing breast. It was found that a regular use of external breast prosthesis involving also wearing it every night results in smaller changes in body posture [13].

Śliwiński [14] and Dobosz et al. [11] also paid attention to significant changes in body posture and the change in the function of spine in women after mastectomy.

However, the available literature does not offer en exhaustive comparative study of women after mastectomy and healthy women, with a large number of subjects and a comprehensive analysis of many features of body posture depending on the degree of involvement of the subject in rehabilitation. The authors have undertaken such a study.

The aim of the study is:

- the analysis of selected features of body posture in women after mastectomy,
- determining differences between body posture of women after mastectomy and the posture of healthy women at a similar age,
- determining the effects of rehabilitation physical exercises on body posture in women after mastectomy.

Material and methods

The following basic groups of subjects were selected for the analysis:

- group A – 85 women after mastectomy (*Tab. 1*),
- group B – 85 healthy women (*Tab. 2*).

The groups of these sizes were examined once. Fourty women from group A were additionally examined twice at six-monthly intervals. In total they were examined three times; in this way group C was formed. Some of them, 20 to be exact, systematically attended rehabilitation classes. The remaining 20 took part in rehabilitation unsystematically or did not attend this type of classes at all.

Group C was divided into:

- group Ce – 20 women after mastectomy systematically taking part in rehabilitation exercises,
- group Cn – 20 women after mastectomy exercising irregularly or not exercising at all.

24 of them underwent a left-sided operation, 15 a right-sided one, and one underwent the operation on both sides.

The examinations were carried out using photogrametric assessment of body posture which is based on the use of Moire topography [12,15,16]. This method involves taking measurements on the basis of computer photography of a subject's body and the use of the phenomenon of moire pattern. Obtaining spatial image is possible thanks to the device "projecting" lines on the subject's back which fall at various angles and are distorted. The image is recorded and analysed by a computer programme. During the examinations the women were standing.

From numerous results of measurements and calculations (in frontal, sagittal and transverse planes) obtained as a result of photogrametric assessment of body posture the following were selected for the analysis:

in the frontal plane

– trunk inclination angle (TIA) from the perpendicular in degrees, that is an angle in frontal plane between the perpendicular and the straight line going through points $C_7 – S_1$,

– maximum deviation of the line of spinous processes from the perpendicular (UK) in mm,

– difference in distance of lower angles of scapulas from the spine (OL) in mm,

– difference in the height of lower angles of scapulas (UL) in degrees,

– inclination angle of the line of shoulders from the level (SLA) in degrees,

in transverse plane, in degrees

– difference in depth of lower angles of scapulas (UB) (assessment of twisting),

– pelvis twist angle (PTA),

in sagittal plane, in degrees

– trunk leaning angle (TLA), that is an angle between the perpendicular and the line $C_7 - S_1$ in the sagittal plane,

– α angle that is an angle of inclination from the perpendicular of lumbosacral spine,

– β angle that is an angle of inclination from the perpendicular of thoracolumbar spine,

– γ angle that is an angle of inclination from the perpendicular of upper thoracic section,

– δ angle that is the total of α, β, and γ angles.

Additionally, results of measurements and calculations of such features as pelvis inclination angle (PIA), thoracic kyphosis angle (TKA), lumbar lordosis angle (LLA) and the ratio of the depth of thoracic kyphosis to its length (TKR) were analysed.

The normality of the distribution of analysed features of body posture was examined. Both normal distributions of the examined features and distributions not in line with normal distribution were found. For this reason in statistical analysis nonparametric tests were used. In tables where results of examinations with a distribution non-compliant with a normal distribution were presented, apart from arithmetic mean also the median and quartiles were shown.

The relation of each of the examined features to basic parameters was examined such as a women's age at the time of examination, a woman's age at the time of operation, time which elapsed from the operation in years, side of amputation (left, right), regularity of rehabilitation, using external breast prosthesis at night, weight and height.

The percentages of results within the norm and with a slight and significant deviation from the norm were specified.

Methodology of rehabilitation exercises

Women in the Ce group were regularly (twice a week) attending rehabilitation classes in a gym. Motion activity lasted about 60 minutes. The programme of therapeutic rehabilitation included:

- increasing or maintaining mobility of shoulder joint on the operated side,
- increasing or maintaining muscle strength of the upper limb on the operated side,
- correction of faulty posture which arose as a result of amputation,
- balancing the strength of postural muscles and developing postural endurance,
- increasing the efficiency of respiratory system,
- prevention of lymph stasis in the limb and in the area of operation,
- improvement of physical efficiency and body fitness,
- effect on the mind in order to achieve adaptation to changed living conditions.

In the rehabilitation of the examined women after mastectomy exercises based on isotonic contraction and short isometric tension were mainly used. Apart from active exercises proper also active exercises without pressure (in suspension) were used, as long as functional abilities of the women allowed it. In individual cases (with weakened muscles, significant limitations of mobility, soreness in the shoulder girdle or other complications) passive or led exercises were used. In exercises low positions were mainly used, since physical exercises in high position (standing) are the greatest burden to the circulatory system, in particular its venous part. Low isolated positions force the subjects to perform proper movements, and do not allow compensating for a limited mobility of shoulder girdle with movement of neighbouring joints (for example using the spine).

Since in women after mastectomy the static and body symmetry are disturbed, scoliosis arises, kypholordosis is changed [11-14,17], it is important to locate the place where effects on the spine are exerted according to the steering rule. Control from above (of upper limbs) and from below (lower limbs) is used. In breathing exercises special attention is paid to breathing route – upper-costal, diaphragmatic and mixed) and to teaching correct breathing rhythm. The aim of breathing exercises is to improve pulmonary ventilation after the operation, gradual stretching of the scar and pressure on the cistern of chyle and abdominal part of thoracic duct (squeezing the lymph out of them towards the head).

During therapeutic rehabilitation educational effects were considered relating to the patient's behaviour at home, using antioedematous prophylaxis and making women realise the significance of physical activity in the prevention of secondary malignant disease.

Results

Trunk inclination angle (**TIA**) informs about the inclination of the trunk to the left or to the right in the frontal plane. For 85 women after mastectomy it varied from 0.0 to 5.2 degrees ($\overline{X}$=1.53°, med=1.2°). For 67 of them the trunk was deviated to the left, for 17 to the right, for one it was vertical. In healthy women 66 left deviations, 13 right deviations and 6 cases of vertical position of the trunk were noted. The results of these examinations indicate clearly that in approximately 78% of women, both healthy and after mastectomy, the trunk is deviated to the left in the frontal plane.

Deviations of TIA from the perpendicular which do not exceed 1.5° were considered normal, those of 3° were considered as slightly deviated from the norm, and those greater than 3° as strongly deviating from the norm (*Tab. 3*).

For 85 women after mastectomy 60% of results were normal, 30.6% of results were slightly deviated from the norm and 9.4% of results were significantly deviated from the norm. Searching for a relation between TIA and basic parameters listed in the "Methods" section only a relation between the direction of TIA (trunk deviated to the left or right) and the age of the subject during operation was found. Women operated at an older age more frequently have their trunks deviated to the right: Spearman's R=0.30 where p=0.006. This is not, however, linked to the side of operation.

Table 3. **Size of TIA and its relation to normative values in healthy women and in women after mastectomy in three examinations (in degrees)**

TIA Group	1st examination			2nd examination		3rd examination	
	B	Ce	Cn	Ce	Cn	Ce	Cn
$\overline{X}$	1.24	1.74	1.37	1.42	1.84	1.83	1.74
min-max	0-3.6	0.3-3.4	0.3-3.3	0-3.1	0-0.4	0-4.3	0.4-3.7
SD	0.84	1.00	0.96	0.94	1.19	1.24	1.12
v (%)	67.9	57.5	70.2	66.3	64.6	67.8	64.3
			% of results				
Normal	63.5	55	50	50	60	45	50
slight deviation	32.9	40	45	30	30	35	25
large deviation	3.5	5	5	20	10	20	25

Table 4. **Value of UK and its relation to normative values in healthy women and in women after mastectomy in three examinations (mm)**

UK Group	1st examination			2nd examination		3rd examination	
	B	Ce	Cn	Ce	Cn	Ce	Cn
$\overline{X}$	4.14	5.30	4.37	4.60	4.44	4.69	5.16
min-max	0-10.4	1.8-10.4	1.1-9.3	1.1-9.4	1.1-9.7	1.2-19.6	1.2-12.6
SD	2.1	2.7	2.1	2.5	2.4	3.9	3.2
v [%]	50.0	50.2	47.2	54.8	54.3	83.4	62.4
			% of results				
normal	70.6	55	70	60	65	75	55
slight deviation	28.2	35	30	40	35	20	40
large deviation	1.2	10	0	0	0	5	5

40 women after mastectomy who were examined three times were characterised by a greater value of TIA, thus a spine more deviated to the side than healthy women. This adverse effect was intensified for women not exercising regularly (Cn group).

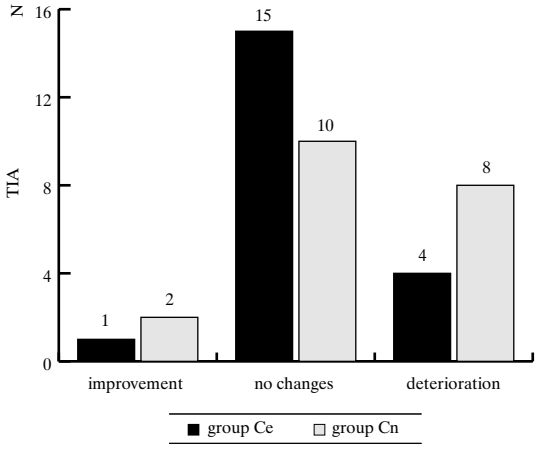
In the group of non-exercising women more cases of deterioration, that is increased TIA between the 1st and the 3rd examination, was found (*Fig. 1*).

A maximum deviation of the line of spinous processes from the perpendicular in the frontal plane **(UK)** informs about the existence of scoliosis even in case of compensating for it and ultimately vertical position of the spine. So there is a possibility of a high value of UK with TIA equal to zero.

In 85 women after mastectomy (group A) the arithmetic mean of UK was 5.0 mm (med 4.3, min-max: 1.1-12.1, Q_1-Q_3: 3.1-6.5), and in healthy women 4.1 mm (*Tab. 4*). The values of UK of up to 5 mm were considered as normal, those up to 10 mm as slightly deviated from the norm and those exceeding 10 mm as strongly deviated from the norm.

In group A 61.2% of UK were normal, 32.9% were slightly deviated and 5.9% strongly deviated. The value of UK did not differ in a statistically significant way between groups A and B. In group A value of UK was related to the use of an external breast prosthesis at night; the subjects who used them were characterised by a lower UK (Spearman's R=-0.23, p=0.030). The location of the spinous process most removed from the perpendicular on the length of the spine was related in group A only to the age of a woman at the time of the examination (R=0.281, p=0.0097). In women older at the time of the examination UK was more frequently located on lower parts of the spine (section Th_7 to Th_{12} or lumbar vertebra), and in younger women on the section Th_1 to Th_6. Such regularity was not found in group B. In group A there were 52 UK results to the left and 33 to the right. Similarly in group B there were 50 UK to the left, 34 to the right and for one subject the spine was straight at the whole length. It can be said that similar to TIA though in smaller percentage, UK is more often directed to the left. No relation between the direction of UK and the side of operation was found.

Figure 1. **Changes in TIA between the 1st and the 3rd examination of women after mastectomy**



In women in Ce group a tendency to lowering of the UK was observed during exercises (*Tab. 4*) and in women in Cn group

Figure 2. **Number of subjects for whom improvement, deterioration or no change in UK was noted in three examinations (between the 1st and the 3rd examination)**

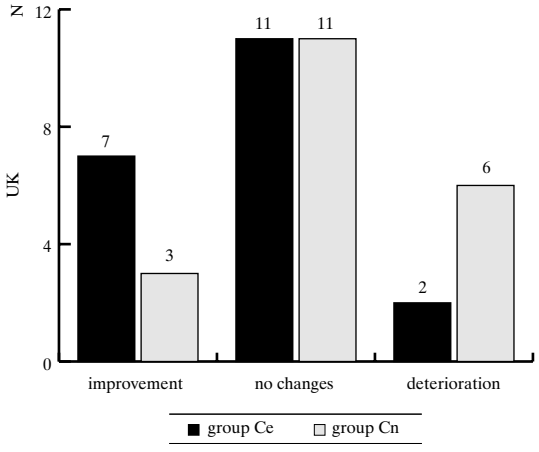


Figure 3. **Number of subjects in whom improvement, deterioration or no changes in OL were noted during three examinations (between the 1st and 3rd examination)**

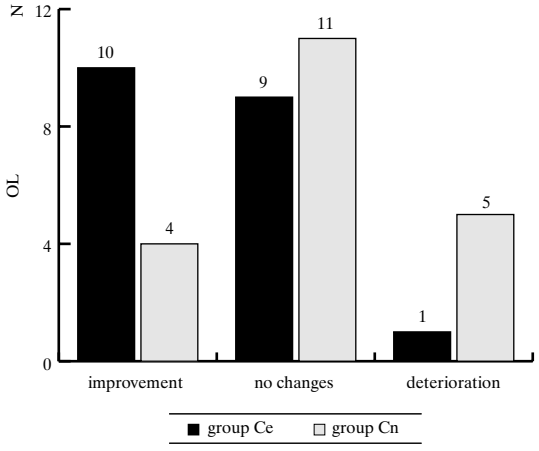


Table 5. **Value of OL and its relation to normative values in healthy women and in women after mastectomy in three examinations (mm)**

OL Group	1st examination			2nd examination		3rd examination	
	B	Ce	Cn	Ce	Cn	Ce	Cn
$\bar{X}$	8.0	15.6	10.9	8.1	7.5	10.0	10.2
min-max	0.0-22.9	0-32.9	1.0-27.3	0-22.7	0-24.3	0.5-27.8	0.5-20.2
SD	5.5	9.1	7.2	6.8	6.4	7.4	5.2
v (%)	68.3	58.6	66.3	83.7	86.3	74.2	51.2
				% of results			
normal	62.3	25	50	75	70	65	35
slightly deviated	34.1	45	40	15	25	25	60
strongly deviated	3.5	30	10	10	5	10	5

– the increase of the UK value. These changes were not always statistically significant.

In group Ce during rehabilitation exercises a number of subjects for whom improvement in UK increased, and the number of subjects for whom UK deteriorated, decreased. A reverse phenomenon was noted in group Cn (*Fig. 2*).

The difference in distance between lower angles of scapulas from the spine (**OL**) informs about the asymmetry of position of scapulas in relation to the spine. In group A it does not show normal distribution. It reaches the arithmetic mean of 11.7 mm (med=10.2 mm, min-max: 0.0-33.5, Q_1-Q_3: 5.1-17.6). The values of OL up to 10 mm were considered as normal, up to 20 mm as slightly exceeding the norm, and higher than 20 mm as strongly deviated from the norm. In the results of group A 48.2% of results were normal, 32.9% were slightly deviated and 18.8% were strongly deviated (*Fig. 3*).

OL does not display a relation with any basic features. It differs in a statistically significant way from group B to the disadvantage of group A (value of Mann-Whitney U test, U=2736 where p=0.0063). In 53 women after mastectomy the right scapula is further from the spine than the left one, in 2 both scapulas are at the same distance from the spine, and in 30 the left scapula is further from the spine than the right one. These numbers are similar in group B (50, 4, 31, respectively).

In group C the level of OL is varied depending on the research subgroup and time of examination (*Tab. 5*).

In group Ce during rehabilitation an improvement in OL was observed in half of all subjects. This result was not analysed statistically due to the small number of patients in groups Ce and Cn, but for the practice of the motional rehabilitation such improvement is highly important.

The difference in the height of lower angles of scapulas (**UL**) informs about asymmetry of scapulas in the frontal plane. In group A the distribution of results was not in line with normal distribution. The arithmetic mean was 2.9°, and the median 1.7° (min-max: 0.0-10.7, Q_1–Q_3: 0.9-4.2). Slight but statistically significant relation between the side of amputation and the direction of asymmetry of scapula position was found (Spearman's R=0.22, p=0.044). The scapula on the operated side is located higher. Mann-Whitney U test did not show a difference in UL between groups A and B. UL deviations and deviations of other discussed features that are SLA and UB, which did not exceed 1.5° were considered as normal, those of 3° were considered as a slightly deviated from the norm and larger than 3° as strongly deviated from the norm.

In group A 45.9% of normal results, 16.5% of results slightly deviated from the norm and 37.6% results with a strong deviation were found. Higher left angle of scapula was noted in 43

Figure 4. **Number of subjects for whom improvement, deterioration or no changes in UL were noted in three examinations (between the 1st and the 3rd examination)**

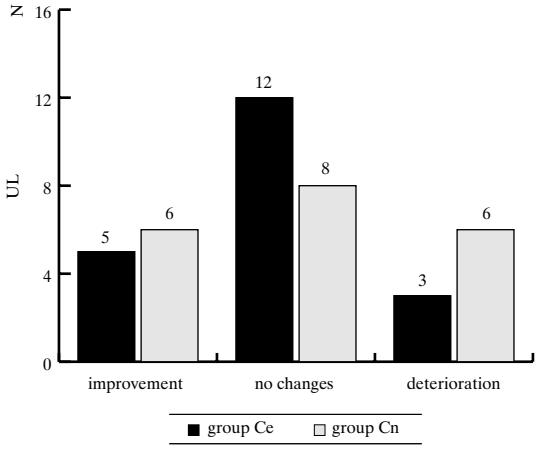


Figure 5. **Number of subjects in whom improvement, deterioration or no change in SLA was noted in three examinations (between the 1st and the 3rd examination)**

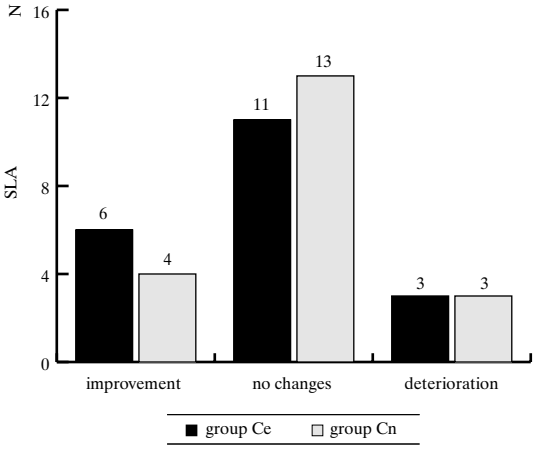


Table 6. **Value of UL and its relation to normative values in healthy women and in women after mastectomy in three examinations (degrees)**

UL Group		1st examination		2nd examination		3rd examination	
	B	Ce	Cn	Ce	Cn	Ce	Cn
$\overline{X}$	1.91	2.03	2.61	2.26	2.55	1.92	2.52
med	1.2	1.65	1.2	2.15	1.8	1.45	1.8
min-max	0-6.6	0-6.5	0-10.7	0-7.1	0-7.3	0-5.2	0-7.7
Q_1	0.9	0.8	0.4	0.8	1.4	0.95	0.9
Q_3	3.1	3.6	4.3	2.95	3.05	5.2	3.25
				% of results			
normal	55.3	45	55	40	25	55	35
slightly deviated	18.8	25	10	40	50	30	30
strongly deviated	25.9	30	35	20	25	15	35

Table 7. **Value of SLA and its relation to normative values in healthy women and women after mastectomy in three examinations (degrees)**

SLA Group		1st examination		2nd examination		3rd examination	
	B	Ce	Cn	Ce	Cn	Ce	Cn
$\overline{X}$	0.91	1.52	1.41	1.33	1.29	1.22	1.42
min-max	0-3.7	0-3.0	0.4-5.1	0-2.7	0-4.5	0-3.5	0-5.7
SD	0.77	0.85	1.15	0.79	1.03	1.03	1.38
v (%)	84.7	56.0	81.5	59.4	79.8	84.4	97.3
				% of results			
normal	82.4	45	70	55	60	70	70
slightly deviated	15.3	55	20	45	35	20	20
strongly deviated	2.4	0	10	0	5	10	10

women in group A and 35 women in group B. Higher right angle of scapula was found in 30 women after mastectomy and in 36 healthy women. Other women had both angles of scapulas at the same level.

In group Cn the value of UL was the least beneficial among all subjects (*Tab. 6*, *Fig. 4*).

Shoulder line angle (**SLA**) informs about deviations of the shoulder line from level in the frontal plane.

In group A it was characterised by a distribution not in line with Gauss' curve. The arithmetic mean of SLA in group A was 1.6° (med=1.3°, min-max 0-6.1, Q_1-Q_3 0.8-2.1). The left shoulder was higher for 40 women after mastectomy and 30 healthy women. The right shoulder was higher for 41 women in group A and 43 women in group B. For other subjects the shoulders were level. In group A 58.8% of normal results, 32.9% of results with slight deviation and 8.2% of results with a strong deviation were found. Mann-Whitney U test indicated a significant difference between SLA values in groups A and B (U=2314, p=0.0000). No relations between the value and size of SLA and basic features were found.

Healthy women were characterised by a lower average SLA and a larger percentage of normal results or results with a slight

Table 8. **Value of PTA and its relation to normative values in healthy women and in women after mastectomy in three examinations (degrees)**

PTA Group		1st examination		2nd examination		3rd examination	
	B	Ce	Cn	Ce	Cn	Ce	Cn
$\overline{X}$	2.69	3.9	3.2	4.1	3.3	5.4	3.8
min-max	0.0-15.2	0.0-21.5	0.0-7.1	0.0-16.7	0.0-9.1	0.0-16.3	0.0-17.2
med	2.2	3.1	3.4	3.3	2.7	4.4	2.1
Q_1	0.8	1.2	1.3	1.5	1.0	2.7	1.2
Q_3	3.9	4.6	4.8	5.0	4.5	6.4	5.2
				% of results			
normal	68.2	50	50	45	55	25	60
slightly deviated	23.5	35	35	35	30	45	20
strongly deviated	8.2	15	15	20	15	30	20

deviation than women after mastectomy. The largest number of subjects with a significant deviation of SLA from the norm was in group C (*Tab. 7*, *Fig. 5*).

The difference in the value of SLA between healthy women and group Ce was statistically significant in the first examination (Mann-Whitney U test, U=490, p=0.003). In the next examinations it became insignificant, which would indicate the improvement of this feature as a result of rehabilitation.

However, the assessment based on of shifting women to another category in the period between the 1st and the 3rd examination did not display differences between groups Ce and Cn.

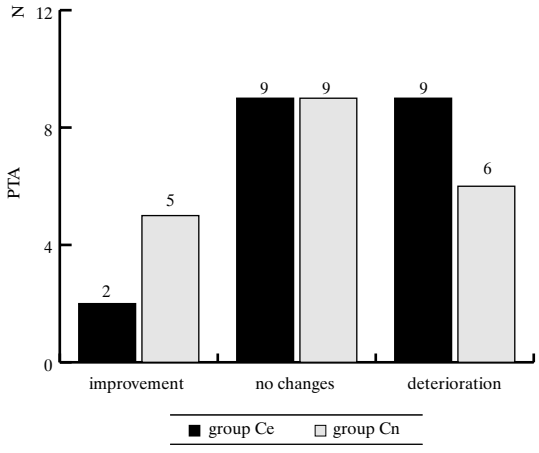
The difference in the depth of lower angles of scapulas informing about their twisting (**UB**) very significantly differs between groups A and B (Mann-Whitney U test, U=2555.5, p<0.001 where $\overline{X}$=3.7° in group A and $\overline{X}$= 2.3° in group B). However, in the group of women after mastectomy it does not show any relation to basic features. In 69 of them the left angle of scapula was more convex than the right one, in 14 the right one was more convex, and in 2 both scapulas were in the same position. Similar division of results was found in group B: 60, 18 and 7, respectively. The changeability of this feature in groups Ce and Cn did not indicate any statistically significant differences.

Pelvis twisting angle (**PTA**) informs about the difference in the depth of position of right and left posterior superior iliac spines, that is about their shift in relation to each other in the transverse plane.

In group A PTA had an average value of 3.9° and a similar value of the median of 3.7° (min-max 0.0-21.5, Q_1-$Q_{3\ 1.1}$-5.4). In 51 women in this group left posterior superior iliac spine was on a larger convexity than the right one, a reverse phenomenon was found in 28 subjects. In 6 women the pelvis was not twisted. In healthy women the same data amounted to: 48, 28 and 9, respectively, so they were very similar.

In group A the value of PTA does not correlate to any basic feature. However, the direction of PTA correlates to the operation age R=0.22, p=0.0399. In subjects operated at an older age the right side the pelvis is moved backwards. The value of PTA in women after mastectomy was higher than in healthy women (Mann-Whitney U test, U=2899, p=0.026). The results of measurements of PTA and the TLA discussed below, which do not exceed 3° were considered as normal, those up to 6° were considered as slightly deviated from the norm, and those exceeding 6° as strongly deviated from the norm. In group A 45.9% of results within the norm, 34.1% results with a slight deviation and 20% with a strong deviation from the norm were found. This is a much worse result than for healthy women (*Tab. 8*, *Fig. 6*).

Figure 6. **Number of subjects for whom improvement, deterioration or no changes in PTA were noted in three examinations (between the 1st and 3rd examination)**



Trunk leaning angle (**TLA**) informs about the leaning of the body forward or backward in the sagittal plane. If points C_7 and S_1 are situated on the same vertical line, TLA equals zero. In groups A and B distribution of results of TLA was in line with normal distribution. In group A TLA was related to body weight and height; taller and heavier women were more leaning forward (correlation coefficient R=-0.33 for weight and R=-0.28 for height with p<0.05). In group B it was related to height: taller women were more leaning forward (R=-0.30, p<0.05).

The comparison of groups A and B with a Student's test for independent samples indicated variation (t=2.8 where p=0.005). Women after mastectomy were more leaning forward ($\overline{X}$=-1.08°, SD=3.1°) than healthy women ($\overline{X}$=0.31°, SD=3.3°). In absolute numbers there were more subjects leaning forward among them.

Table 9. **Value of TLA and its relation to normative values in healthy women and in women after mastectomy in three examinations (degrees)**

TLA Group	1st examination			2nd examination		3rd examination	
	B	Ce	Cn	Ce	Cn	Ce	Cn
$\overline{X}$	2.7	2.2	3.0	2.3	2.5	3.2	3.0
min-max	0.0-7.1	0.2-4.5	0.0-6.2	0.0-6.7	0.4-6.1	0.3-5.7	0.0-10.2
SD	1.8	1.2	2.0	1.8	1.8	1.6	2.8
v (%)	65.7	53.8	68.7	76.7	73.0	49.9	94.1
			% of results				
normal	63.5	80	60	65	60	50	65
slightly deviated	30.6	20	25	30	35	50	25
strongly deviated	5.9	0	15	5	5	0	10

Table 10. **Value of α angle in healthy women and women after mastectomy in three examinations**

α angle Group	1st examination			2nd examination		3rd examination	
	B	Ce	Cn	Ce	Cn	Ce	Cn
$\overline{X}$	6.1	6.9	8.2	7.6	6.4	9.4	7.2
min-max	0.0-18.1	0.0-24.5	0.0-16.3	0.0-14.9	0.0-14.2	0.0-19.6	0.0-20.5
med	5.3	5.8	8.5	7.0	6.2	10.3	7.8
Q_1	2.8	3.1	3.3	4.6	3.2	7.1	3.1
Q_3	8.9	9.7	12.2	12.0	9.5	13.0	8.8

Accepting as the most correct posture the situation when points C_7 and S_1 are in one vertical line, the number of normal results and results beyond the norm was established. Forward or backward deviation from the perpendicular up to 3° was considered as within norm, up to 6° as slight deviation and above 6° as strong deviation from the norm.

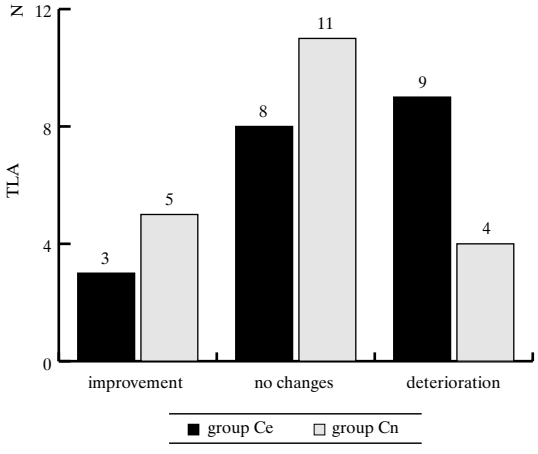
In group A 58.8% of results were normal, 37.6% of results displayed a slight deviation and 3.5% of results – a strong deviation from the norm. In group B there were more results within the norm (*Tab. 9*, *Fig. 7*).

Examining the relation between the time which elapsed from the mastectomy and the direction of TLA (leaning backward or forward) indicates that a recent operation is related to inclination forward and an operation long time ago to backward leaning.

When observing the direction of changes in TLA in group C (leaning forward or backward as a result of regular rehabilitation) ANOVA Friedman's test and Kendall's compatibility coefficient were used. In group Ce slight but statistically significant differentiation of this feature was noted in three examinations; the arithmetic means increased gradually, that is the subjects (group Ce) leaned forward more and more with each examination (Chi squared ANOVA=6.6, $p<0.038$). Women in group Cn leaned forward more and more as well, but it was not statistically significant.

The value of TLA is affected by proportions between α, β and γ angles. Therefore, the values of individual angles and their total value, that is δ angle were analysed. In group A an average value of α angle was 7.5° (with min-max 0.0-24.5° and SD=5.2°). In women after mastectomy α angle was characterised by a significantly greater changeability than in healthy women (*Tab. 10*). However, Mann-Whitney U test did not display statistically significant differences between distributions of results

Figure 7. **Number of subjects in whom improvement, deterioration or no change in TLA was noted in three examinations (between the 1st and the 3rd examination)**



of measurement of α angle between groups A and B. The values of α angle do not show a relation to any of the basic features. In group Ce α angle showed a similar value between the 1st and the 3rd examination, in group Cn it was decreasing distinctly, however, this difference tested with ANOVA-Friedman test is statistically insignificant.

The values of α, β, γ and δ angles were not assessed in terms of their compliance with normative values which in this case are very difficult to determine (*Tab. 11, 12, 13*).

In all groups β angle was characterised by normal distribution, in group A was on average 9.0° (SD=3.8, min-max 0.0-17.4). Thus, it did not differ significantly from the group of

Table 11. **Value of β angle in healthy women and in women after mastectomy in three examinations**

β angle		1st examination		2nd examination		3rd examination	
Group	B	Ce	Cn	Ce	Cn	Ce	Cn
$\overline{X}$	9.7	10.3	10.0	9.0	10.8	7.9	9.9
min-max	1.8-17.5	5.3-15.1	3.3-17.4	2.0-16.5	3.4-17.0	0.6-12.1	-0.4-16.7
SD	3.5	2.7	3.5	3.5	3.7	3.0	4.9
v (%)	35.1	25.8	34.6	38.6	34.6	37.9	49.5

Table 12. **Value of γ angle in healthy women and in women after mastectomy in three examinations**

γ angle		1st examination		2nd examination		3rd examination	
Group	B	Ce	Cn	Ce	Cn	Ce	Cn
$\overline{X}$	15.8	16.4	17.5	19.6	17.6	19.9	17.7
min-max	2.2-30.8	10.3-24.5	4.1-29.1	8.6-29.3	6.6-24.2	8.2-27.4	9.1-30.1
SD	5.3	4.3	5.2	4.9	4.1	5.4	5.5
v (%)	33.4	26.3	29.5	25.0	23.3	27.3	30.8

Table 13. **Value of δ angle in healthy women and in women after mastectomy in three examinations**

δ angle		1st examination		2nd examination		3rd examination	
Group	B	Ce	Cn	Ce	Cn	Ce	Cn
$\overline{X}$	31.6	33.5	35.7	36.1	34.8	37.2	35.0
min-max	17.2-49.8	24.7-57.1	22.6-52.0	24.5-51.2	25.2-41.4	19.0-55.7	20.4-45.2
SD	6.9	7.4	6.6	7.4	4.3	9.4	6.6
v (%)	21.9	22.0	18.4	20.5	12.5	25.3	18.8

healthy women. In group A its value was related to height in reverse relation (Spearman's R=-0.35, p=0.001). A similar relation was noted in group B (R=-0.33, p=0.0024). In group Ce its value was systematically going down, and these changes are statistically significant (ANOVA Friedman's χ^2=8.3 p<0.016). This significance resulted mainly from the difference between the 1st and the 3rd examination.

The value of γ angle the results of which were also characterised by normal distribution, differed statistically significantly between women after mastectomy and healthy women (Mann-Whitney U test, U=2749, p=0.007). In group A its average value amounted to 18.1° (min-max 4.1-41.5, SD=5.4), it was not related, however, to any of the basic features. The value of this angle increased systematically in subsequent examinations in group Ce (ANOVA Friedman's χ^2=7.7, p<0.02), in group Cn it was stable.

The δ angle is a total of α, β and γ angles. In group A δ angle reached an average value of 34° (min-max 20.6-57.1, SD=7.1). A total value of angles of spinal curvatures in women after mastectomy was higher compared to healthy women (Mann-Whitney U test, U=2939, p<0.036). The value of δ angle was in group A directly proportional to weight (Spearman's R=0.26, p=0.017). In group B no such relation was noted. In group Ce a total value of spinal curvatures was systematically growing, though in a statistically insignificant manner, and in group Cn it remained stable.

From additionally analysed features – pelvis inclination angle, thoracic kyphosis angle, lumbar lordosis angle and **the ratio of the depth of thoracic kyphosis to its length**, the latter one named as **TKR** showed some interesting properties. The values of TKR differed in a statistically significant way between groups A and B (group A $\overline{X}$=0.03, SD=0.016, group B $\overline{X}$=0.05, SD=0.012, Student's t -2.53, p<0.012). The lengths of thoracic kyphosis expressed as a percentage of the whole length of the spine are very similar in groups A and B. Thus, a lower average TKR in women after mastectomy is caused by a smaller depth of thoracic kyphosis in them ($\overline{X}$=7.8 mm) compared to healthy women ($\overline{X}$=11.6 mm). Regular participation in rehabilitation classes (group Ce) resulted in decrease (flattening) of thoracic kyphosis in a statistically significant way (arithmetic mean of TKR decreased in subsequent examinations 0.04, 0.02, -0.24, ANOVA Friedman's χ^2=8.9, p<0.011). The results of the examinations allowed noting that in group Ce the length of lordosis in subsequent examinations increased, which resulted in shortening of thoracic kyphosis. Although statistically not significant, this change could be observed in group Ce, it did not occur in group Cn, though. The value of TKR correlated to a woman's age at the time of operation (R=-0.22, p<0.05).

Discussion

The results of studies presented here make it possible to specify the features of body posture in women after mastectomy and compare them to the body posture of healthy women, which was the aim of the work. It can be stated that the body posture of women after mastectomy compared to healthy women is characterised by the following, statistically significant alterations:

– greater trunk inclination angle,
– greater symmetry of scapula position,
– higher position in the frontal plane of the scapula on the operated side,
– much greater angle of shoulder line which means that shoulder are more deviated from the level in frontal plane,
– much greater difference in the depth of lower scapula angles,
– greater angle of pelvis twisting,
– greater forward leaning of the trunk,
– greater total value of angles of spinal curvatures,
– lower ration of the depth of thoracic kyphosis to its length and lower depth of thoracic kyphosis. Thus, greater forward leaning of trunk in women after mastectomy is caused by a greater α angle in them,
– directly proportional relation between the value of δ angle and weight,
– a tendency to increase of the γ angle and large interpersonal changeability of the α angle.

These results are in agreement with the results of few papers published up to date and concerning this topic [5]. However, the presented results of the trunk leaning angle (TLA) measurements differ in this article from what Dobosz et al. [6] presented in the paper presenting body posture of women after mastectomy. Like us, other authors, have not found any link between side of surgery and the direction of asymmetry of body posture alterations.

The relation between the results of examinations of body posture and features called basic were not strong and did not allow isolating one feature determining the body posture of women after mastectomy. However, it was found statistically significant that:

– there is a slight, although statistically significant relation between the side of amputation and the direction of asymmetry of position of scapulas; the scapula in the operated side is higher,
– women operated at an older age more frequently have their trunks deviated to the right, and the right side of their pelvises is moved backwards,
– the location of the spinous process furthest deviated from the perpendicular on the length of the spine was related in group A with the age of a women at the time of the examination. In women older at the time of examination UK most frequently was located on lower sections of the spine (section Th_7 to Th_{12} or lumbar vertebra), and in younger women on section Th_1 do Th_6,
– recent operation is related to forward leaning of the trunk, and operation a long time ago with backward leaning of the trunk; it should be assumed that leaning of the trunk forward a short time after the operation is an analgesic and protective position which passes with time,
– the value of UK in group A was related to the use of external breast prosthesis at night; the subjects who used it were characterised by a lower UK.

The comparison of the changes in features of body posture in groups Ce and Cn allows us to note the following positive effects of rehabilitation in subjects who exercise regularly:

– maintaining TIA on a stable level,
– decrease that is improvement in UK, OL, SLA.

PTA is a feature which does not show improvement or even shows deterioration in women after mastectomy who exercise regularly. Since features related to the position of scapulas and shoulder girdle improved during exercises it may be assumed that this took place at a cost of compensation achieved by a change in the position of pelvis. This opinion should be, however, supported by a study of individual cases.

The increase in PTA in Ce group seems to be a negative effect of rehabilitation. If compensation is a result of anti-scoliosis exercises, more careful stabilisation of pelvis during all exercises affecting the spine, scapulas and shoulder girdle is needed. Their position should not be corrected at the costs of compensation by twisting the pelvis. Thus, emphasis should be placed on isolated positions during physical exercises.

It should be stressed that the groups Ce and Cn were not numerous, which negatively affected the statistical significance. And yet the results obtained three times when women from the group Ce exercised regularly are of great value for the practice of motional therapy.

The results of the examinations informing about the changes in spine angles (α, β, γ and δ) and its natural curvatures (lordosis, kyphosis) are ambiguous. Women in group Ce during a year of exercises leaned forward more than group Cn where smaller leaning was also noted. In any case this is not a positive phenomenon. Thus, more exercises of back muscles – the thoracic part (decreasing the γ angle) and exercises stretching the chest should be introduced to rehabilitation.

Conclusions

In the study great changes in body posture of women after mastectomy were noted compared to healthy women at similar age. This phenomenon was so far only partly recognized [20]; however, it was not described in detail. It should be considered in what way the negative effects of mastectomy could be reduced. Hawro et al. [9] write about the significance of early, postoperation rehabilitation for the reduction of effects of mastectomy. The results of the studies are an argument for putting an emphasis on early, and then on long-term, continuous rehabilitation. This may be a way to stop later irregularities. Starting rehabilitation too late can lead to changes which are difficult to reverse.

Great changes in body posture are also an argument for wider introduction of breast reconstruction operations in Poland, since the use of breast implants gives better results in maintaining body symmetry [14]. Though asymmetry may also take place with the use of implant [14], its intensity is much smaller.

Lymphatic oedema also contributes to the intensification of disorders in body posture [3]. Rehabilitation should always be combined with antioedematous prophylaxis.

Undoubtedly taking part in rehabilitation, apart from its influence on somatic features of the subjects, has a very significant positive effect on their minds [2]. This is an additional important argument for popularization of rehabilitation in women after mastectomy.

Future research on the motional rehabilitation of women after mastectomy should head in two directions. First, the alterations in body posture should be monitored during regular physical exercises. There is an urgent need to work out an exact program of those exercises that improve particularly difficult features in the body posture. Patients should also be protected against compensatory changes. Another research topic is based on observation of alterations in body posture and their prevention in those women on whom new surgical techniques, e.g. breast reconstruction, were conducted.

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The comparison of effect of catechins and green tea extract on oxidative modification of LDL *in vitro*

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Abstract

Purpose: Green tea due to its content of catechins reveals strong antioxidative activity, which is manifested among others by its ability to inhibit free radical generation, scavenge free radicals as well as chelate transition metal ions that catalyse free radical reactions. The influence of green tea extract, epicatechin (EC), epicatechin galate (ECG) as well as epigallocatechin galate (EGCG) on oxidative modifications of LDL of human blood serum has been examined in the present study.

Materials and methods: This influence has been evaluated by measurement of the concentration of first products of lipid peroxidation – conjugated dienes and lipid hydroperoxides as well as by determining tryptophan and dityrosine content – the markers of protein oxidative modification.

Results: Catechins and green tea abilities to protect lipophilic antioxidant – α-tocopherol against oxidation have been also examined. The results reveal that peroxidation of LDL is markedly prevented by green tea extract and in a slightly weaker way by catechins (EGCG in particular), which is manifested by a decrease in concentration of conjugated dienes, lipid hydroperoxides, MDA, dityrosine and by an increase in tryptophan content. Both green tea as well as catechins (EGCG in particular) have been also revealed to prevent decrease in concentration of α-tocopherol in oxidating conditions.

Conclusions: It can be assumed that green tea and to a lesser degree catechins, protecting the basic antioxidant of LDL-α-tocopherol, prevent oxidative modification of LDL.

Key words: LDL, green tea, catechins, lipid peroxidation, protein oxidative modifications, α-tocopherol.

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Introduction

A key early event in the development of atherosclerosis is the oxidation of low density lipoprotein via different mechanisms including free radical reactions with both protein and lipid components. The LDL particle (~2.5 MDA) consists of an apolar core of cholesteryl esters (42%) and triglicerides (6%), surrounded by a monolayer of phospholipids (22%), unestrified cholesterol (8%) and one molecule of apolipoprotein B-100 (4536 amino acids, 550 kDa) (22%) [1]. Thus, each LDL particle contains about 1600 molecules of cholesteryl ester, 170 of trigliceryde, 700 of phospholipid and 600 molecules of free cholesterol [2]. Human LDL contains a number of antioxidants that inhibit lipid oxidation, with α-tocopherol the most abudant (~6 α-tocopherol molecules per LDL particle) and other antioxidants (e.g. carotenoids, ubiquinol-10) present in much lower abudance [2].

LDL phospholipids consist of polyunsaturated fatty acids. They have at least one methylene group between double bindings. Elimination of hydrogen atom from methylene groups and displace of double bindings occurs under the influence of oxidizing factors, hydroxyl radical in particular [3]. This transformation results in generation of conjugated dienes which next react with particle oxygen with generation of superoxide radicals. If unpaired electron is localized at the end of a double bindings system it is reduced to lipid hydroperoxides. Lipid hydroperoxides are simple lipid peroxidation products and are relatively constant in the case of transition metal ions absence [4]. Further alterations of peroxidation products, occurring among others on the β-elimination way, lead to decomposition of polyunsaturated fatty acid residues and to generation of few and/or several carbon fragments. Lipid peroxidation final products include cycle ethers, hydroperoxides and carbonyl compounds, such as aldehydes being the most important group among them, including malondialdehyde and 4-hydroxynoneal [5]. Compounds that are lipid peroxidation products may be used as markers of intensification of this process [6]. Among lipid peroxidation products, significant reactivity is revealed particularly by α, β-unsaturated aldehydes, which easily

react with proteins and DNA, and therefore are characterized by toxic and mutagenic properties [7]. Among lipid peroxidation products, 4-hydroxynoneal reveals the strongest toxic activity, whereas malondialdehyde is the most mutagenic [8,9].

The mechanisms by which metal ions stimulate LDL oxidation are poorly understood. Reduced copper ions (Cu^{2+}) catalyze the decomposition of lipid peroxides into alkoxyl radical, a potent oxidizing species [10]:

$Cu^{2+} + LOOH = LO^{\bullet} + HO^{-} + Cu^{+}$

The hydroperoxide-derived radicals next are scavenged by antioxidants. They can also react with polyunsaturated fatty acids (LH) to form carbon-centered radicals ($L^{\cdot}$) that initiate the radical chain reaction of lipid peroxidation [10]. The reaction cycle continues until antioxidants or radical-radical cross-linking reactions terminate lipid peroxidation.

$LO^{\bullet} + LH = LOH + L^{\cdot}$

One possible mechanism of LDL oxidation is the conversion of α-tocopherol to α-tocopherol radical [11,12]. The radical then attack a polyunsaturated fatty acid to initiate lipid peroxidation.

α-tocopherol $+ Cu^{2+} =$ α-tocopherol radical $+ Cu^{+} + H^{+}$

α-tocopherol radical $+ LH =$ α-tocopherol $+ L^{\cdot}$.

Reduction of bound Cu^{2+} to Cu^{+} by endogenous α-tocopherol appears to be a key step in initiating LDL lipid peroxidation [11]. This leads to the counter-intuitive proposal that α-tocopherol, normally considered to be an antioxidant, can promote the peroxidation of LDL lipid [12].

Free oxygen radicals may also cause oxidative modification of protein part of LDL – apolipoprotein B-100 [13]. It may undergo oxidative modification as a result of direct reaction of free radicals with amino acid residues or in reaction with carbonyl compounds, generated during degradation of phospholipid peroxidation products – mainly malondialdehyde and 4-hydroxynonenal [14]. These kinds of modifications may even lead to apolipoprotein B-100 particle degradation [2].

To prevent these type of reactions, compounds revealing antioxidative properties, which are demonstrated among others by ability to reduce the amount of free radicals, may be applied. More and more attention has been recently paid to compounds of natural origin. Of particular importance is green tea, which contains abundance of catechins – compounds which reveal strong antioxidative properties [15]. Catechins may reduce of possibilities of occurring oxidative modifications of biologically important compounds, including lipids or proteins.

Therefore the present study has determined the influence of catechins and green tea extract on peroxidation of LDL, through measurement of concentration of succesive lipids peroxidation products – conjugated dienes, lipid hydroperoxides and malondialdehyde as well as oxidative protein modification products – tryptophan and dityrosine. Moreover, the influence of catechins and green tea extract on the protection of the basic lipophoilic antioxidant – α-tocopherol has been examined.

Materials and methods

Determination of catechins in green tea

(-)-epicatechin, (-)-epigallocatechin, (-)-epigallocatechin gallate were obtained from Sigma-Aldrich Chemie Gmbh (Steincheim, Germany). Green tea – Camellia sinensis (Linnaeus) O. Kuntze (standard research blends-lyophilized extract) was provided by TJ Lipton (Englewood Cliffs, NJ). Green tea extract contained epigalloctechin gallate (97 mg/g dried extract), epigallocatechin (82 mg/g dried extract), epicatechin (90 mg/l), epicatechin gallate (15 mg/g dried extract) and caffeic acid (10 mg/g dried extract), determined by HPLC [16].

Isolation of LDL from serum

The LDL fraction was isolated from extraplacental human serum by precipitate method in the presence of heparin and manganese chloride [17]. The protein content in the LDL fraction was measured by the Lowry method [18].

LDL oxidation *in vitro*

The stock LDL solution (5.3 mg protein/ml) was diluted by Tris-HCl buffer (final concentration 0.2 mol/l, pH 7.4) to obtain concentration 150 μg protein/ml). The oxidative modification of LDL was initiated by addition of $CuSO_4$ solution (final concentration 5 μmol/l). To counteract oxidative modification of LDL, epicatechin (EC), epigallocatechine (EGC) and epigallocatechine gallate (EGCG) (final concentration 10 μmol/l) as well as green tea extract (final concentration 3%) were added to samples 5 minutes after Cu^{2+} ions addition. LDL solution (150 μg protein/ml) in Tris-HCl buffer (final concentration 0.2 mol/l, pH 7.4) was the control sample. All samples were incubated for 1, 2, 4, 6, 18, 22 and 30 hours at 37°C. The levels of lipid peroxidation products – conjugated dienes, lipid hydroperoxides, malondialdehyde and of protein oxidative modification products – dityrosine and tryptophan and of α-tocopherol were measured in all samples.

Determination of conjugated dienes

In this method dienes were extracted with chloroform-methanol mixture (2:1; v:v) and were quantitated by their 234 nm absorbance in cyclohexane, relative to cyclohexane blank [19].

Determination of lipid hydroperoxides

Lipid hydroperoxides were determined by a sensitive and specific HPLC method involving chemical conversion 1-naphthyldiphenylphosphine into 1-naphthyldiphenylphosphine oxide, which was injected on RP 18 column eluated by methanol-water mixture (8:2; v:v) at 35°C , the detection was carried at $\lambda = 292$ nm [20].

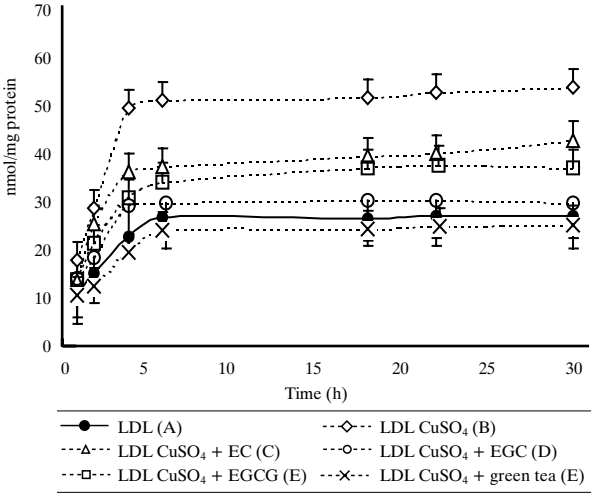
Determination of malondialdehyde

The procedure involves formation MDA with thiobarbituric acid (TBA) adducts and the separation of TBA-MDA adducts on RP 18 column with spectrofluorometric quantification at 532 nm excitation and 553 nm emission. The separation was carried by mixture 40% methanol and 60% phosphorate buffer at pH 7.0 [21].

Determination dityrosine and tryptophan

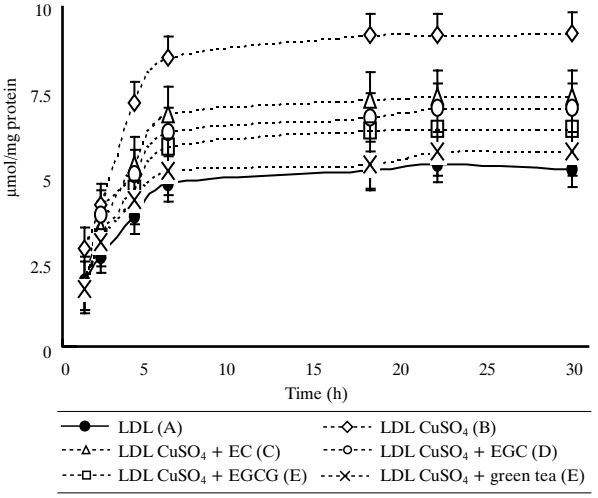
Tryptophan and dityrosine were measured with a spectrofluorometer Hitachi 2500. Signal intensity was calibrated against 0.1 mg/ml quinine sulfate solution in sulfuric acid with fluorescence was assumed as a unit. Fluorescence emission at

Figure 1. **The effect of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and green tea extract on the level of conjugated dienes in human LDL fraction in presence of 5 μM Cu^{2+} (n=6)**



The values for $p<0.05$ were considered significant:
2 h: A – B, C; B – A, D, F
4 h: A – B, C; B – A, C, D, E, F
6 h: A – B, C, E; B – A, C, D, E, F
18 h: A – B, C, E; B – A, C, D, E, F
22 h: A – B, C, E; B – A, C, D, E, F
30 h: A – B, C, E; B – A, C, D, E, F

Figure 2. **The effect of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and green tea extract on the level of lipid hydroperoxides in human LDL fraction in presence of 5 μM Cu^{2+} (n=6)**



The values for $p<0.05$ were considered significant:
2 h: A – B; B – A, F
4 h: A – B, C, D; B – A, C, D, E, F
6 h: A – B, C, D, E; B – A, C, D, E, F
18 h: A – B, C, E; B – A, C, D, E, F
22 h: A – B, C, E; B – A, C, D, E, F
30 h: A – B, C, E; B – A, C, D, E, F

338 nm (288 nm excitation) was used as a reflection of tryptophan content, dityrosine content was estimated at 325 nm excitation and 420 nm emission [22].

Determination of α-tocopherol by HPLC

The lipid fraction was extracted from LDL solutions by hexane, and the organic layer was next dryied under nitrogen. The residue was dissolved in ethanol and injected on RP 18 column. The separation was carried by mixture 95% methanol and 5% water with spectrophotometric detection at 294 nm [23,24].

Statistical analysis

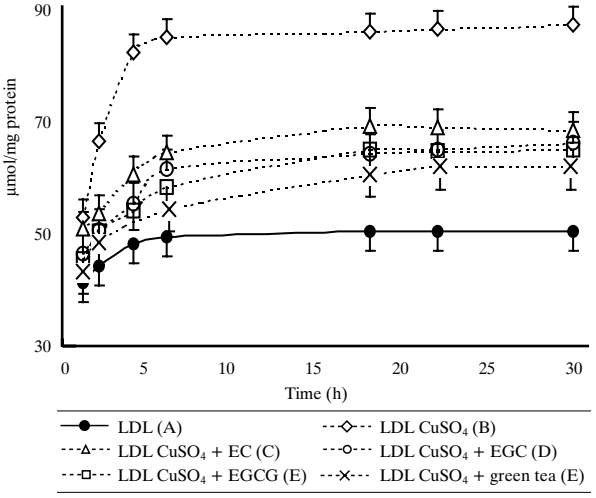
The data obtained in this study are expressed as mean ±SD. The data were analysed by use of standard statistical analyses, one way Student's test for multiple comparisons to determine significance between different groups. The values for $p<0.05$ were considered as significant.

Results

Incubation of LDL with Cu^{2+} ions results in lipid peroxidation. This is manifested by an increase in the amount of primary products of this process – conjugated dienes (up to about 98%) and lipid hydroperoxides (up to about 63%) in comparsion to LDL solution (*Fig. 1* and *Fig. 2*). The level of these compounds increases during 6 hours incubation and after these time its concentration is not changed. However, their level is remarkably lower in the presence of catechins as well as green tea extract. Influenced by green tea, the content of conjugate dienes is decreased by about 54%, and lipid hydroperoxides by about 48% compared to oxidized LDL. The level of final product of lipid peroxidation – malondialdehyde increases up to about 76% during 6 hours in the presence of Cu^{2+} ions, and its concentration is not changed after this time (*Fig. 3*). Catechins and green tea extract remarkably prevent an increase in MDA concentration in LDL solution with Cu^{2+} ions. Lipid peroxidation is the most effectively prevented by epigallocatechin gallate (35% decrease in lipid hydroperoxides concentration and 45% decrease in malondialdehyde concentration in relation to LDL solution with Cu^{2+} ions) and by epigallocatechin (43% decrease in conjugated dienes concentration in relation to LDL solution with Cu^{2+} ions), and the most weakly by epicatechin (26% decrease in conjugated dienes concentration, 10% decrease in lipid hydroperoxides concentration and 25% decrease in malondialdehyde concentration in relation to oxidized LDL).

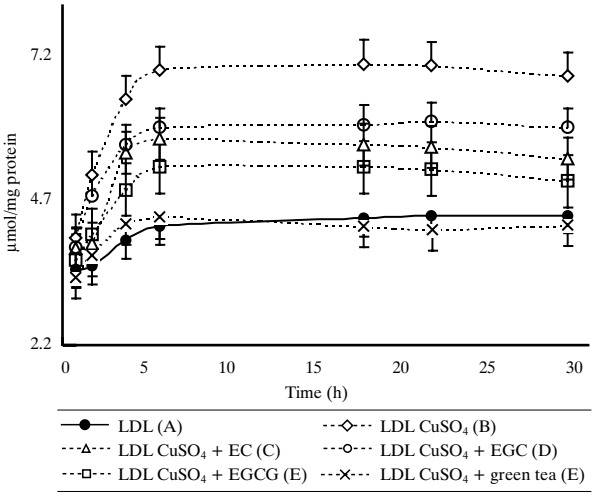
Incubation of LDL solution with Cu^{2+} ions also causes oxidative modifications of protein part of LDL – apolipoprotein B-100. It is manifested by the increase in dityrosine concentration (*Fig. 4*) and by the decrease in tryptophan concentration (*Fig. 5*). Dityrosine content increases by about 93% after 6 hours incubation, and level of tryptophan is decreased by about 41% in relation to the solution containing LDL only. Such significant changes in values of the above parameters do not occur in the presence of catechins and green tea. The supplementation of green tea completely prevents the increase in dityrosine level in LDL fraction due to Cu^{2+} ions oxidation. In the oxidized LDL solution containing green tea dityrosine level is significantly

***Figure 3*. The effect of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and green tea extract on the level of malondialdehyde in human LDL fraction in presence of 5 μM Cu^{2+} (n=6)**



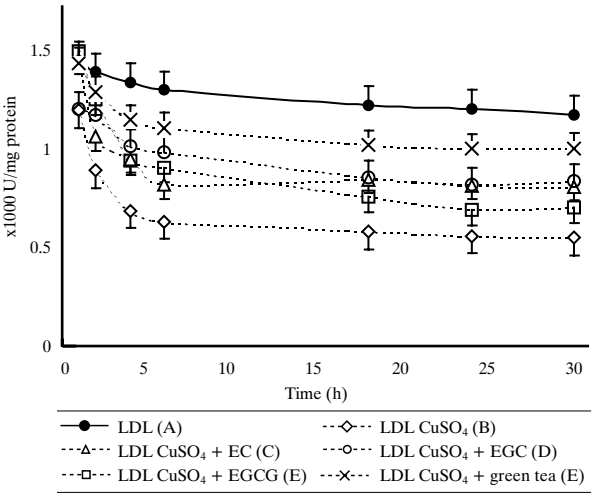
The values for $p<0.05$ were considered significant:
2 h: A – B, C; B – A, D, F
4 h: A – B, C, D; B – A, C, D, E, F
6 h: A – B, C, D, E; B – A, C, D, E, F
18 h: A – B, C, E; B – A, C, D, E, F
22 h: A – B, C, E; B – A, C, D, E, F
30 h: A – B, C, E; B – A, C, D, E, F

***Figure 4*. The effect of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and green tea extract on the level of dityrosine in human LDL fraction in presence of 5 μM Cu^{2+} (n=6)**



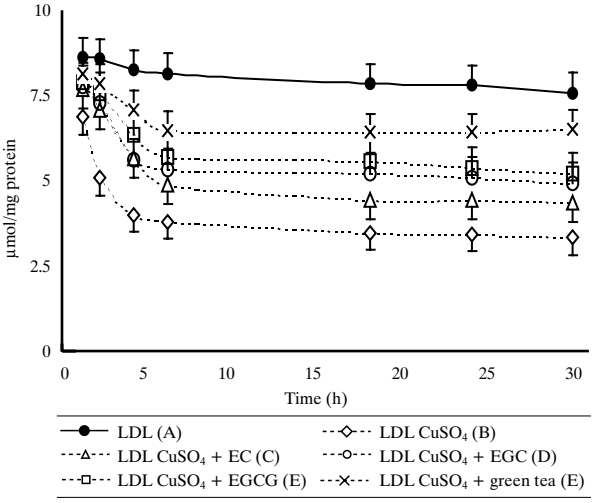
The values for $p<0.05$ were considered significant:
4 h: A – B, C, D; B – A, C, D, E, F
6 h: A – B, C, D, E; B – A, C, D, E, F
18 h: A – B, C, D, E; B – A, C, D, E, F
22 h: A – B, C, D, E; B – A, C, D, E, F
30 h: A – B, C, D, E; B – A, C, D, E, F

***Figure 5*. The effect of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and green tea extract on the level of tryptophane in human LDL fraction in presence of 5 μM Cu^{2+} (n=6)**



The values for $p<0.05$ were considered significant:
2 h: A – B, C; B – A, F
4 h: A – B, C; B – A, D, E, F
6 h: A – B, C, D, E; B – A, C, D, E, F
18 h: A – B, C, E; B – A, C, D, F
22 h: A – B, C, E; B – A, C, D, F
30 h: A – B, C, E; B – A, C, D, F

***Figure 6*. The effect of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) i green tea extract on level of α-tocopherol in human LDL fraction in presence of 5 μM Cu^{2+} (n=6)**



The values for $p<0.05$ were considered significant:
2 h: A – B; B – A, F
4 h: A – B, C, D; B – A, D, E, F
6 h: A – B, C, D, E; B – A, D, E, F
18 h: A – B, C, E; B – A, D, E, F
22 h: A – B, C, E; B – A, D, E, F
30 h: A – B, C, E; B – A, D, E, F

decreased (by 57%) while tryptophan content is increased (by about 38%) in relation to concentration of these compounds in oxidized LDL. Among the examined catechins, epigallocatechin gallate reveals the strongest protective action against generation of dityrosine (31% decrease in its concentration) and epigallocatechin against tryptophan decrease (40% increase in tryptophan concentration in relation to LDL solution with Cu^{2+} ions), whereas epicatechin is characterised by the weak-

est protective action against this process (about 8% decrease in dityrosine concentration and 37% increase in tryptophan concentration in relation to oxidized LDL).

Influenced by incubation of LDL with Cu^{2+} ions decrease in α-tocopherol content occurred (*Fig. 6*). The amount of this compound is reduced throughout 4 hours incubation, after which it reaches the constant value. Addition Cu^{2+} ions into the LDL solution causes significant (by about 48%) decrease in α-tocopherol content. Among the applied antioxidants, the green tea extract and epigallocatechin gallate most effectively protect against decrease in α-tocopherol concentration (by 80% and 62% respectively). The catechin that reveals the weakest protective properties against decrease in α-tocopherol content is epicatechin because in its presence concentration of α-tocopherol is increased by about 12% only in relation to LDL solution with Cu^{2+} ions.

Discussion

The present study has revealed that Cu^{2+} ions cause LDL peroxidation. It is manifested by intensified oxidative modification of the lipid and protein LDL part through increase in concentration of conjugated dienes, lipid hydroperoxides, malondialdehyde, dityrosine as well as through decrease in the content of tryptophane and α-tocopherol.

For *in vitro* studies of oxidized LDL, oxidation by copper is frequently used and produces LDL and is similar to LDL oxidized by cells [13]. Copper is more potent than iron in its ability to oxidize LDL *in vitro* [13]. Copper ions are known to induce the formation of radicals in the aqueous phase and they can affect with amino acids of apolipoprotein B-100 [13,25].

A number of antioxidants may act in partly by inhibiting the binding of copper to LDL. Our results are in agreement with previous authors [26] who have demonstrated an antioxidant effect with catechins and particularly green tea extract during copper-mediated oxidation of LDL. Antioxidant capacity of catechins is related to their localization in the LDL particle, as well as to their chemical structures. Catechins contain both lipophylic and hydrophylic moieties, but are mostly hydrophylic and can act as potent inhibitors of LDL oxidation via several mechanisms: scavenging of free radicals by acting as reducing agents, as hydrogen atom donating molecules; chelation of transition metal ions, thereby reducing the metals capacity to generate free radicals; and as well as sparing of vitamin E in the LDL particle, thus protecting LDL from oxidation. Catechins possess penthahydroxypolyphenol structure. Strong antixidative properties of a molecule are due to the presence of at least 5 hydroxyl groups and the double bond of pyrone ring. Such structure has been identified as important for both chelating and radical scavenging activity. The hither to existing examinations have proved, that epigallocatechin gallate and epicatechin gallate reveal the strongest antioxidative properties, and epicatechin – the weaker. The present results confirm these dependences. It has been found that the green tea extract reveals stronger protective activity against lipid peroxidation than single catechins. Therefore the essential property of the catechins is their synergism.

Catechins may inhibit the lipid peroxidation process by scavenging free radicals (mainly owing to orto-dihydroxyphenol structure), as well as by regeneration of α-tocopherol. The present study has revealed, that green tea catechins remarkably prevent decrease in α-tocopherol concentration under the influence of Cu^{2+} ions activity. The literature data suggest that green tea catechins (EGCG in particular) regenerate tocopherol radical to α-tocopherol through the ability to release hydrogen atom to lipid radicals [27]. Moreover, catechins having lower reducing potentials than oxygen free radicals may prevent reduction of α-tocopherol concentration through scavenging oxygen radicals such as hydroxyl radical, superoxide anion, peroxide and lipid radicals [27-31], which occurred in the presence of Cu^{2+} ions. Catechins ability to scavenge radicals is also connected with its di- or trihydroxyl structure of the phenyl ring, which secures stability for radical forms. The present study has demonstrated that epigallocatechin gallate is the most effective catechin, which prevented lipids peroxidation the strongest.

Studying the causes of efficacy of antioxidative activity of EGCG it has been found that it is connected with possessing 3 hydroxyl groups of the phenyl ring [32]. Antioxidative activity of EGCG is also intensified by hydroxyl group estrification in position 3, by gallate acid, since as it has recently been revealed, the gallate acid residue may react with lipids radicals [33].

The present study has demonstrated that also protein part of LDL – apolipoprotein B-100 undergoes oxidative modifications under the influence of Cu^{2+} ions, what was manifested by an increase in dityrosine concentration and decrease in tryptophan concentration. The oxidation products of the lipid components of LDL have been studied extensively, less is known about the oxidation products of apolipoprotein B-100. Metal-catalyzed oxidations have been studied. The functional groups formed by metal-catalyzed oxidations that have been characterized to date have been primarily aldehydes and ketones, but hydroxylated phenylalanine, tyrosine, leucine and valine residues have been reported, along with dityrosine as a product of oxidation [34]. It was shown as well as that Cu^{2+} catalyzes protein oxidation through close binding to tryptophan residues with a site-specific redox reaction that yelds a tryptophan radical and Cu^{+}. Moreover, copper ions are able to bind to the histidine residues on apolipoprotein B-100. The number of binding sites reported varies widely over 2 orders of magnitude [13]. During metal-catalyzed oxidations a redox-active transition metal (e.g. Cu^{2+}/Cu^{+}) binds to a metal-binding site on the protein and Cu^{+} – protein complex reacts with H_2O_2 to produce reactive oxygen species that resemble $HO^{\bullet}$. The reactive oxygen species react with molecular structures in the immediate vicinity of the metal-binding site, resulting in the formation of aldehyde or ketone derivatives. Our results additionally demonstrate that catechins and green tea reveal protective properties in relation to apolipoprotein B-100. They partly prevent dityrosine generation and decrease in tryptophane concentration.

The similar time of studied antioxidants action results from similar antioxidant capacity and similar structure of their particles.

Our results give evidence for the effectiveness of green tea in counteracting changes in antioxidative system and in the lipid peroxidation process that are intensified in the presence of Cu^{2+} ions. It suggests the posibility of its application for neutralizing the consequences of free radicals overproduction in pathological cases.

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Changes in platelet CD 62P expression and soluble P-selectin concentration in surgically treated colorectal carcinoma

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Abstract

Purpose: The aim of the present study was to assess the effect of tumor advancement and surgery treatment on P-selectin expression (CD 62P), level of sP-selectin and platelet count.

Material and methods: The study involved 27 colorectal cancer patients (CRC). They were divided into two groups: group B1 – 18 patients (T2-3N0M0) and group B2 – 9 patients (T2-4N+M0). In CRC patients the blood was collected three times: 1) before surgery (A0), 2) 3 days after surgery and 3) 12 days after surgery.

Results: The results obtained showed that CD 62P expression in CRC patients was twice higher (5.36%) than in control (2.58%) ($p<0.001$). The level of sP-selectin concentration in both groups (B1 – 74.22 ng/mL and B2 – 70.33 ng/mL) was significantly higher than in control (46.01 ng/mL) ($p<0.001$). There was no significant differences in CD 62P expression, plasma sP-selectin concentration and in PLT count between group B1 and B2. Three days after surgery in both groups of patients we observed decreased CD 62P expression and sP-selectin level compared to the results before surgery ($p<0.05$). Twelve days after surgery we found an increase in the CD 62P--positive platelets and sP-selectin in group B1 and B2. We found positive correlation between plasma sP-selectin concentration and PLT count in CRC.

Conclusions: In the current study on colorectal cancer we observed platelet hyperactivation, irrespective of tumor clinical advancement. Surgical procedure, in the early period following radical tumor resection, does not totally eliminate platelet activation *in vivo*.

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Key words: colorectal cancer, blood platelets, CD 62P, sP-selectin, platelet counts.

Introduction

P-selectin (CD 62P, also known as GMP-140), 140 kDa, belongs to the family of adhesion molecules. It is rich in cystein and binds in resting platelets to the membranes of α granules and Weibel-Palade bodies of endothelial cells [1]. Upon platelet activation and release from α granules, P-selectin is translocated into the platelet surface. The exposure of surface P-selectin is temporary as it is soon "shed" to the plasma and is found in a soluble form (sP-selectin) there [2]. Michelson showed that the elevated concentration of sP-selectin was accompanied by a drop in CD 62P expression [2]. The percentage of activated platelets with P-selectin expression assessed by means of flow cytometry is regarded as the "golden standard" of platelet activation [3].

P-selectin, as an adhesion molecule, plays a key role in the interaction of platelets with other cells. A small amount of PSGL-1 is also present on platelet surface and can mediate platelet-endothelium interactions *in vivo* [4]. P-selectin released from endothelial cell granules initiates leukocyte and platelet rolling on the vessel wall, which is an important process in both inflammation and hemostasis [5]. The latest studies have provided some evidence for the presence of CD 24 ligand on cancer cells, recognized by P-selectin [6,7]. Discovery of the CD 24 molecule on the cells of certain types of cancer indicates that P-selectin can mediate tumor cell interactions with platelets, leukocytes and endothelium *in vitro* [8,9]. This fact seems to be of significance in understanding the role of adhesion molecules and platelets in the formation of tumor metastasis. Cancer cell binding by platelets is an important observation – platelet aggregations formed around cancer cells play a protective role against the host immune system; in consequence, they prolong cell survival time and promote formation of metastases [10]. Moreover, platelets protect tumor cells against the environment and are

the source of growth factor, such as platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), that stimulate growth of cancer cells [11].

Quantitative thrombocytopenia or thrombocytosis, and blood platelet abnormalities found in cancers may lead to disturbances in hemostasis [12]. In neoplastic disease, platelets are capable of hyperactivation *in vivo*. An increase has been found in sP-selectin concentration or CD 62P expression in patients with thrombotic diseases, atherosclerosis and in neoplastic disease – in lung, renal, breast and colon cancer [13-17]. These abnormalities include hemorrhagic complications – a likely consequence of platelet dysfunction, vessel infiltration by cancer cells or DIC. However, the enhanced platelet activity and thrombocytosis can increase the risk of thrombosis, which is one of the most frequent causes of death due to cancer [12].

Hemostatic disorders, including platelet abnormalities in which both platelet count and function are affected are commonly found in colorectal cancer. Many studies dealing with the effect of tumor clinical advancement, presence of metastases and the influence of surgery treatment on morphological and functional parameters of platelets have yielded controversial issues. Therefore, we decided to investigate the problem in colorectal cancer patients.

The aim of our study was to assess the effect of tumor advancement on P-selectin expression (CD 62P) and plasma level of soluble P-selectin and platelet count as well as the effect of surgery on the parameters examined in colorectal cancer patients. An attempt was also made to find any correlations between these parameters in colorectal cancer patients.

Material and methods

Patients

The study included 27 patients (10 women and 17 men; aged 52-80 years) with colorectal cancer treated surgically in II Department of General Surgery, University Hospital of Białystok. They were enrolled to the study between November 2003 and September 2004.

The diagnosis of colorectal cancer was based on clinical symptoms, and endoscopy (colonoscopy), radiology or CT. All the patients had colorectal adenocarcinoma, confirmed by histopathological examination.

Colorectal cancer patients were divided into two groups, according to TNM classification:

- Group B1 – 18 patients without metastases (7 women and 11 men; mean 67.5 years) – T2-3N0M0 (stage I and II)
- Group B2 – 9 patients with lymph node involvement (4 women and 5 men, mean 61 years) – T2-4N+M0 (stage III).

At the time of the study, there were no patients with distant metastases (M+, grade IV) of colorectal cancer.

Colorectal cancer patients awaiting surgery, who gave their written consent to the study, were recruited. The patients underwent surgical procedure of tumor resection with subsequent regional lymphadenoctomy.

Before and after surgical treatment the patients received no cytostatics nor chemo- or radiotherapy. Those who took aspirin or other platelet function affecting drugs (steroidal, anti-inflammatory) in the preceding week were excluded from the study. All the patients were given a preventive dose of heparin (LMWH) – *Enoxiparinum natricum* (Clexane) 20-40 mg once daily for 7-10 days, with the first injection administered a day before the procedure. If required, the heparin dose was increased up to 80 mg daily.

The study was approved by Bioethics Committee, Medical University of Białystok according to Guidelines for Good Clinical Practice.

Control group

Control group (C) consisted of 21 healthy subjects, men and women aged 42-74 years. They had no taken aspirin and other antiplatelet drugs during the preceding week.

Material

The blood collected from the vein to tubes in a closed system, without stasis was used for analysis. 2 ml blood samples were collected for anticoagulant EDTA-K2 to assess platelet count and 3.6 ml blood samples to evaluate P-selectin expression and level of sP-selectin using 3.2% sodium citrate as anticoagulant.

In CRC patients blood was collected three times: 1) before surgery (A0); 2) 3 days after surgery (A1), to assess the effect of surgery and the related trauma on P-selectin expression and thrombocytopoiesis, the effect of a number of factors that accompany the procedure (hypoxia, acidosis, interleukins and growth factors) and systemic hemostatic balance, 3) 12 days after surgery (A2), taking into consideration mean survival time and platelet turnover.

In control group (C) blood was collected once.

Methods

Flow cytometry Surface P-selectin expression (% of CD 62P positive platelets) was assessed using monoclonal antibodies CD 62P/RPE (DAKO, Denmark), on a flow cytometer EPICS XL Coulter according to the protocol of European Working Group on Clinical Cell Analysis [18].

Additionally, monoclonal antibodies CD 61/FITC (DAKO, Denmark) were used to identify platelets among other morphological components. IgG1/RPE (DAKO, Denmark) antibodies served as negative control antibodies.

Venous blood was taken to plastic tubes containing sodium citrate. To determine P-selectin expression on platelets *in vivo* immediately after blood collecting, a 100 µl sample was placed in a test tube containing 1 ml of 1% paraformaldehyde in PBS (without Ca^{2+} and Mg^{2+}) and incubated for 30 minutes at room temperature. Then, 20 µl alignots of the suspension were placed into two test tubes containing the following antibody sets: 1) CD 61/FITC+ IgG1/RPE, 2) CD 61/FITC+ CD 62P/RPE. This was followed by 30 min incubation in darkness and after addition of 1 ml PBS the samples were analyzed on a flow cytometer.

Signal from 10000 platelets was analyzed in each measurement. The results were presented as the percentage of CD 62P positive platelets (% CD 62P+), i.e. activated platelets with release reaction.

Assay of soluble P-selectin Blood samples were collected in citrate-containing tubes. Samples were centrifuged at 1000 x g

Table 1. **P-selectin expression and soluble P-selectin concentration and platelet count in colorectal cancer patients and in control group**

***a*) P-selectin expression (CD 62P % positive)**

	n	A0	A1	A2	p
CRC group	27	5.36±1.58**	3.13±0.65	4.40±1.85**	
B1 group	18	5.69±2.09**	2.87±0.88	4.64±1.9**	A0:A1*, A1:A2**
B2 group	9	4.66±1.20**	2.45±0.50	4.41±2.55*	A0:A1*
Control group	21	2.58±1.36			

***b*) sP-selectin concentration (ng/mL)**

	n	A0	A1	A2	p
CRC group	27	68.7±20.56**	55.8*±21.57	81.6±26.07 **	A0:A1*, A1:A2**
B1 group	18	74.22±28.80**	62.80±33.30	92.00±21.12**	A0:A2*, A1:A2**
B2 group	9	70.33±25.09**	52.00±21.91	63.14±13.07*	A0:A1*
Control group	21	46.01±8.09			

***c*) PLT count (x 10^3/μl)**

	n	A0	A1	A2	p
CRC group	27	262.8±64.2	245.6±80.9	356.2±142.,2**	
B1 group	18	259.6±72.5	234.5±70.5	339.4±54.0	A0:A2, A1:A2*
B2 group	9	271.5±109.7	270.1±141.4	394.6±161.0**	A0:A2,A1:A2*
Control group	21	232.6±32.0			

Results are expressed as mean ±SD; **p<0.001 vs C, *p<0,05 vs C; A0 – before surgery; A1 – 3 days after; A2 – 12 days after surgery; CRC – colorectal cancer patients; B1 – tumor without metastases; B2 – tumor with lymph node metastases

within 30 minutes. Then plasma were kept frozen at -80°C, and thawed before determination of sP-selectin. sP-selectin levels were determined using the Quantikine human sP-selectin immunoassay kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Platelet count PLT count was determined on a hematological analyzer ADVIA 120 (Bayer). Platelet count in healthy subjects is 150-350 x 10^3/μl.

Statistical analysis

The results were subjected to statistical analysis using non-parametric U-Mann-Whitney tests to compare data between the groups B1 and B2 CRC patients and with control findings (group C) before surgery. Wilcoxon's test was applied to compare differences in the parameters in time (before and 3 and 12 days after surgery). And Pearson correlation coefficient was calculated.

Results were presented as mean ±SD. Differences were considered significant for $p<0.05$ and highly statistically significant for $p<0.001$. Statistical package SPSS 8.0 PL was used for calculations.

Results

Platelet CD 62P expression in colorectal cancer patients before surgery (A0) was over twice higher than in healthy subjects ($p<0.001$) (*Tab. 1a*). The percentage of CD 62P-positive platelets was higher in metastasis-free patients – group B1 as compared to patients with lymph node involvement – group B2, but the differences were not statistically significant. However, highly statistically significant were the differences in the expression of CD 62P in the study groups in comparison to controls ($p<0.001$) (*Tab. 1a*). The level of soluble P-selectin was slightly higher in study patients than in control group (68.7 ng/mL vs 46.01 ng/mL; $p<0.001$) (*Tab. 1b*). It was higher in group B1 than in group B2, although the difference was not statistically significant ($p<0.067$) (*Tab. 1b*). In patients with colorectal cancer (A0), PLT count was higher than in control group, although the differences were statistically insignificant. No differences in PLT count were also found with regard to tumor advancement – group B1 and group B2 (*Tab. 1c*).

Three days after surgery (A1) colorectal cancer patients showed significantly decreased platelet CD 62P expression, which approached control values. In both study groups the percentage of CD 62-positive platelets was significantly lower as compared to the expression before surgery (A0:A1, $p<0.05$) (*Tab. 1a*). The level of sP-selectin in both groups was decreased as compared to the level before surgery, but it was still higher than in controls (*Tab. 1b*). At the same time, in both study groups, platelet count was slightly reduced in comparison to that noted prior to surgery (*Tab. 1c*).

Twelve days after surgery (A2), we found an increase in the percentage of CD 62P-positive platelets both in the group of colorectal cancer patients and in clinical advancement subgroups (B1 and B2) (*Tab. 1a*). The percentage values of CD 62P in the group B1 were lower as compared to that group before surgery (*Tab. 1a*). At the same time we observed increased con-

centration of sP-selectin in CRC patients, especially in group B1. Differences in sP-selectin between the groups were statistically significant (*Tab. 1b*). Moreover, a statistically significant increase was observed in PLT count in the study groups as compared to the values obtained in the patients 3 days after (A1) and before the procedure (A0; $p<0.05$) (*Tab. 1b*). A significant increase in platelet count – thrombocytosis ($394.6x10^3/\mu l$) was noted in the group B2, i.e. in patients with metastases to the lymph nodes; it was statistically significant both in comparison to PLT before surgery (A0) and 3 days after the surgery (A1) (A0:A2, A1:A2; $p<0.05$) (*Tab. 1c*).

The relationship between plasma sP-selectin and CD 62P expression and PLT count in CRC patients was also evaluated. We found no statistical correlation between sP-selectin and CD 62P. However, we observed positive correlations between sP-selectin and PLT count in group B1 ($r=0.6027$, $p<0.023$) and in group B2 ($r=0.8242$, $p<0.006$).

Discussion

Numerous studies point at the occurrence of hyperactive platelets in cancer patients [12,14-16,20]. Upon platelet activation, tumor growth-inducing substances TXB2 and VEGF are released [21], and P-selectin involved in neoplastic spread can be found on platelet surface. On the other hand, a number of factors released by tumors such as cancer procoagulant, thrombin, ADP, tissue factor (TF) are capable of direct platelet activation [22].

We found increased platelet activation *in vivo* in colorectal cancer patients, which was manifested by over twice as high CD 62P expression on platelet surface and elevated level of soluble P-selectin as compared to healthy subjects. Slightly higher expression of CD 62P and higher sP-selectin level were observed in patients with stage I and II carcinoma, in comparison to stage III, the differences being statistically insignificant. Lack of differences between these markers of platelet activation and clinical staging may suggest that tumor causes platelet activation independent of metastasis and could be related to the inflammatory response which is associated with CRC. Some authors obtained different results, perhaps due to differences in the number of study patients – our group was small, while groups of other investigators were large. Findings similar to ours have been reported by Mantur et al. for renal cancer [15]. The author observed an increase in both surface and soluble P-selectin, but found no effect of lymph node involvement on platelet activation in renal cancer patients [15]. However, Ferroni, who studied colorectal cancer, revealed higher sP-selectin level in patients with more advanced carcinoma and distant metastases [17]. Similarly, in lung cancer Roselli found a correlation between the level of sP-selectin and the presence of distant metastases, and as we know many investigators have suggested platelet involvement and direct contribution of P-selectin to the formation of metastases [14,23,24].

We found a correlation between sP-selectin and PLT count but not between the examined markers of platelet activation. It may indicate that sP-selectin is rather derived from blood platelets. This hypothesis could be in agreement with the findings of Ferroni et al., who observed no correlation between sE-selectin and sP-selectin, and suggested that the increased level of sP-selectin originated from platelets rather than endothelial cells due to platelet activation [17]. The same positive correlation between sP-selectin and PLT count was also noted by Fijnheer et al. [25]. According to some authors, sP-selectin is a better and more useful marker of platelet activation, especially in patients with thromboembolic disorders, as compared to cytometric analysis of CD 62P [2,25]. It is likely that P-selectin expression is temporary, and the receptor is then thrown into the circulation and labeled as soluble P-selectin, while the percentage of CD 62P+ returns to baseline level. This hypothesis is based on the observation that an increase in plasma sP-selectin is accompanied by a decrease in CD 62P expression [2].

Data on the effect of surgery on platelet activation in cancer patients are scarce. Our patients underwent resection of colorectal tumor with regional lymphadenectomy. Three days after surgery we noted a significant decrease in CD 62P expression, nearly to the values observed in healthy subjects. Also sP-selectin level was reduced, although not so much as expression of CD 62P, indicating that in the early postoperative period in colorectal cancer patients platelets still show a considerable activation potential. This can be associated with the inflammatory process accompanying the surgical procedure, wound healing and cytokine production (e.g. IL-6 involved in acute phase reaction and stimulating thrombocytopoiesis) [26]. At the same time we observed a decrease in platelet count, most likely caused by the loss of more active platelets due to bleeding or shortened survival time in cancer patients, or caused by heparin administration in the perioperative period. According to some authors, heparin, which is a P- and L-selectin inhibitor, suppresses tumor growth and formation of metastases [27,28]. Experimental studies have demonstrated that even a single heparin dose inhibits platelet interaction with tumor cells, which is P-selectin-mediated [27]. This could explain a considerable drop in CD 62P expression compared to sP-selectin in our patients.

Twelve days after surgery we found enhanced stimulation of thrombocytopoiesis and platelet activation in patients with colorectal cancer. We noted an increase in CD 62P expression and sP-selectin level in both study groups of patients as compared to day 3 and interestingly, we observed statistically significant differences in sP-selectin level between the groups. This may indicate that the procedure had a certain effect on platelet activation, but did not totally eliminate the platelet activating factors. Lower platelet activation indicated by much lower sP-selectin level in stage III patients may be caused by shorter survival time or lower potential of PLT, considering that high-activity platelets have been utilized in the formation of metastases in these patients.

Stimulation of thrombocytopoiesis after surgery has been observed by Folman et al. [29,30]. The authors noted the maximum increase in platelet count between day 7 and day 20 after surgery, associating it with the level of thrombopoietin, major regulator of thrombocytopoiesis [29]. Platelet turnover and production activation due to their intra-surgical exploitation result in the appearance of young platelets in the circulation 12 days after surgery and hence raised platelet count. The young platelets show higher metabolic activity, are more sensitive to

the action of activating factors and have more surface receptors [15].

Based on the expression of surface P-selectin and the level of soluble P-selectin, the current study has revealed that colorectal carcinoma induces intravascular platelet hyperactivation, irrespective of clinical advancement. Positive correlation between sP-selectin and PLT count seems to indicate that sP-selectin may be derived from blood platelets. Surgical procedure exerts a significant effect on the stimulation and platelet count, but in the early period following radical tumor resection it does not eliminate platelet activation *in vivo*.

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Anemia treatment with darbepoetin alpha in pregnant female with chronic renal failure: report of two cases

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Abstract

Pregnancy is a rare finding in females with CRF. It is well known that in these cases pregnancy worsens the renal function and accelerates the beginning of dialysis therapy. Pregnancies in uremic females are complicated by a worsening of anemia as well as hypertension, fluid imbalance, electrolyte difficulties, malnutrition, polyhydramnios and preterm labor or sudden intrauterine death. The use of erythropoiesis stimulating agents (ESA) allows a better hematocrit control. Data concerning anemia treatment in pregnants with CRF and data regarding the use of ESA in these patients is scarce. We report two cases of anemia treatment with darbepoetin alpha in pregnant women with CRF in predialysis period and during the hemodialysis therapy. Both patients during the darbepoetin treatment did not require any blood transfusions and at 32nd and 37th weeks of pregnancy delivered healthy infants. The high darbepoetin doses did not cause any side effects, were well tolerated and safe for both gravida and fetus. The effective anemia treatment in pregnants with CRF improves the prognosis for a successful pregnancy.

Key words: pregnancy, anemia, chronic renal failure, darbepoetin alpha, hemodialysis.

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Introduction

Pregnancies in uremic females are complicated by a worsening of anemia as well as hypertension, fluid imbalance, electrolyte difficulties, malnutrition, polyhydramnios and preterm labor or sudden intrauterine death. The principal problem is chronic anemia. Anemia in pregnant women results from iron defficiency and from hypervolaemia. The iron requirement during pregnancy as well as its absorption from the digestive tract increases systematically, especially in the 3rd trimester [1]. Anemia treatment in a pregnant is based mostly on iron supplementation. In a pregnant with renal failure, the factors that intesify the anemia are inadequate erythropoiesis, folic acid deficiency, hyperparathyroidism and concomitant inflammation. In a pregnant without CRF, the EPO concentration is normal, and it increases systematically during the pregnancy [2]. It has been observed that in dialyzed patients and those treated with EPO, pregnancy causes a considerable increase in the EPO requirement [3]. Data concerning anemia treatment in pregnant women with renal failure and data regarding the use of erythropoiesis stimulating agents (ESA) in these pts is scarce. We report two cases of anemia treatment with darbepoetin alpha in pregnant women with chronic renal failure (CRF). In the first case, treatment with darbepoetin was started during the predialytic period; in the second case – at the beginning of HD treatment.

Case Reports

Patient 1. A 33-year-old female was admitted to the renal clinic in the 16th week of her pregnancy due to symptoms of weakness, dizziness and arterial hypertension. Laboratory data showed haemoglobin 7.73 g/dl, RBC 2.53 T/l, WBC 5.9 G/l with a normal differential count and platelets 222000/mm^3. Serum creatinine was 2.85 mg/dl, blood urea 91.4 mg/dl, total protein 6.05 g/dl and the uric acid – 7.8 mg/dl. The iron levels were low during the pregnancy (40-48 μg/dl); TIBC was 280-300 μg/dl;

TSAT – 14-16%. Antinuclear antibodies (ANAs) and antineutrophilic cytoplasmic antibodies (p/cANCA) were negative. Haematuria (5-8 RBCs) and proteinuria were observed. Urine protein excretion was 2.5-3.0 g/24 h. Her blood pressure was 150/90 mmHg. An ultrasonography of the maternal kidney showed a decrease in kidney dimension to 8 cm. The etiology of CRF was unknown. The patient had regular treatment with methyldopa 400 mg/day and iron per os. An injection of darbepoetin alpha 10 μg once a week subcutaneously was initiated in the 22nd week of the pregnancy and continued throughout her pregnancy. After 5 weeks of treatment, the Hb concentration was 8.86 g/dl and serum creatinine elevated to 3.3 mg/dl. The blood pressure remained on the level of 140/80 mmHg. After another 5 weeks of darbepoetin treatment in unchanged dose, the Hb concentration was 7.7 g/dl and the anemia was well tolerated. The patient did not require any blood transfusion. Ultrasound examinations of the fetus in the 16th, 20th, 27th and 35th weeks of gestation revealed normal intrauterine development. At the 37th week of gestation the patent delivered a 2010-gm healthy infant by spontaneous labour with an Apgar score of 10. The post-partum serum creatinine increased to 5.8 and 6.2 mg/dl, blood urea to 160 mg/dl. As the pregnancy progressed, her requirement for antihypertensives increased. Post partum, her blood pressure was 180/110, and amlodipine 10 mg/day and perindopril 4 mg/day were added. Metabolic acidosis and hypervolaemia were found, and peritoneal dialysis (CAPD) was started. During the two-month period a slow increase in Hb concentration up to 12 g/dl was observed.

Patient 2: A 31-year-old female undergoing haemodialysis (HD), with a 15-year history of IDDM complicated by hypertension and diabetic nephropathy. The pre-pregnancy serum creatinine levels ranged from 2.0 to 3.0 mg/dl. She began HD therapy in the 20th week of gestation when the serum creatinine was 3.15 mg/dl and BUN was 56.0 mg/dl. From the first HD session, a darbepoetin alpha was administrated intravenously once a week. The initial dose was 0.48 μg/kg b.m./week (40 μg), and it increased during the pregnancy to 1.15 μg/kg b.m./week (100 μg). The serum albumin levels decreased, and the patient required several albumin transfusions during the dialysis sessions. The patient received iron, vitamin C, D, folic acid, calcium and zinc during the pregnancy. She was dialyzed with a biocompatible polysulphone high-flux membrane (1.3 m^2) using a bicarbonate bath. The HD dose was increased from 12 to 21 hrs, and the frequency increased from 4 to 6 times per week. Her blood pressure was not stable, and she required changes in her antihypertensive regimen. The Hb level increased from 9.44 to 11.4 g/dl. The patient did not require any blood transfusions during the dialysis treatment. At the 32nd week of gestation the patient delivered a 1730-gm female infant by cesarean section. The Apgar score was 9 and 10 at the 1st and 5th minutes, respectively. The postoperative course was benign, and both mother and baby are currently doing well. The patient continues the dialysis treatment and she is being prepared for kidney and pancreas transplantation.

Discussion

Anemia treatment in pregnant women with renal failure is a matter of interest to researchers. Positive results of anemia treatment with rHuEPO during pregnancy were described in a number of publications [4,5]. Treatment with EPO in pregnants after renal transplantation was equally successful [6,7]. The aim of EPO treatment is to obtain a Hb value on the level of healthy pregnant women (11 to 12 g/dl) [8]. In a number of publications, a relevant increase of gravida's blood cell count and an absence of fetus complications, including malformations that could be possibly related to the treatment, were pointed out [9]. Erythropoietin does not penetrate from the mother's blood into the fetal circulation, which guarantees safety for the fetus. EPO treatment in a pregnant with CRF is a much better alternative than blood transfusion. 26% of pregnants require blood transfusions, irrespective of rHuEPO treatment [10]. In the cases described, there was no need for blood transfusion during the darbepoetin alpha treatment, even in a patient in which the expected Hb value increase during the predialytic period was not obtained. In this case the increase in Hb value was obtained after the labour. Hyporesponsiveness to EPO in pregnants results from hypervolaemia and an apparent decrease in Hb value. Also, in concomitant CRF, the EPO synthesis decreases, which gives reason for its supplementation in pregnant women. Hyporesponsiveness to treatment increases during pregnancy. Diabetic pregnants treated with hemodialysis required a gradual increase of darbepoetin doses, up to 100 μg/week. ESA treatment in pregnants require iron supplementation from the beginning of the pregnancy, preferably intravenously [4]. Darbepoetin alpha stimulates erytropoiesis the same way as rHuEPO. Due to the 3 times longer half-time period and the greater biological activity, compared to rHuEPO, the darbepoetin maintains a constant Hb concentration despite the doses being administrated in longer intervals. This drug may be administrated intravenously as well as subcutaneously, which proves its value and usefulness during the predialytic period as well as during HD-therapy. The use of darbepoetin alpha in anemia treatment in patients with CRF is effective and well tolerated [11]. The described cases prove that the drug is well tolerated and safe for both gravida and fetus. To date, no red cell aplasia (PRCA) has been described during the darbepoetin treatment [11].

Conclusions

Darbepoetin alfa appears to be a safe and effective drug correcting anemia in pregnant females with chronic renal failure. These cases indicate hyporesponsiveness to darbepoetin alpha in uremic females, and the need for using much larger doses than in dialysis patients. The high drug doses did not cause any side effects in either mother or her infant. It demonstrates that anemia treatment with darbepoetin improves the prognosis for a successful pregnancy.

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Thrombomodulin in human gestational tissues: placenta, fetal membranes and myometrium

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Abstract

Purpose: In intervillous space, thrombomodulin (TM) can be a key component of the protein C anticoagulant pathway that controls the balance between coagulation and anticoagulation/ /fibrinolysis via activation of protein C (APC). In our working hypothesis we assume that not only TM from the placenta, but also TM from myometrium might be engaged in this protective mechanism. To determine whether this is potentially possible, we decided to measure TM concentration in placenta and myometrium, and also in fetal membranes.

Material and methods: The study group consisted of 35 parturient women who delivered at term by cesarean section. Strips of placenta, fetal membranes and myometrium, as well as venous blood samples were collected during operation. The tissues were homogenized. TM was measured by immunoenzymatic method (ELISA).The concentration of TM antigen in placenta was 18.76±3.83 ng/mg proteins, in fetal membranes 8.57±1.64 ng/mg proteins and in myometrium 4.72±1.93 ng/mg proteins, while in blood plasma it was 0.063±0.016 ng/mg proteins.

Conclusions: It was shown for the first time that thrombomodulin is present in gestational myometrium and fetal membranes. The results support the hypothesis that not only placental TM, but also myometrial TM can participate in maintaining the fluidity of the blood in utero-placental circulation.

Key words: thrombomodulin, placenta, fetal membranes, myometrium.

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Introduction

Thrombomodulin (TM) is an integral membrane-glycoprotein of 75 kDa of molecular weight which is expressed on the capillary endothelium and therefore its highest concentration is found in densely vascularized organs like heart, lungs and placenta [1]. In placenta, TM is expressed by extravascular and vascular trophoblast as well as by fetal capilary endothelium [2].

TM is a high-affinity receptor for thrombin forming thrombin-thrombomodulin complexes which then activate zymogen protein C to a powerful anticoagulant protein C (APC). Thrombin-thrombomodulin complex can also activate the latent inhibitor of fibrinolysis, e.g. procarboxypeptidase B to carboxypeptidase B producing thrombin activatable fibrinolysis inhibitor (TAFI) [3,4]. Once APC is generated, it binds protein S as a cofactor and can then inactivate factors Va and VIIIa, thus decreasing thrombin generation. The rate of protein C activation by the thrombomodulin-thrombin complex is greatly enhanced when protein C is bound to the endothelial protein C receptor (EPCR) [5,6]. Furthermore, the fibrinolytic effect of TM *in vivo* depends on its concentration in local vasculature [7].

There are two main forms of TM which differ with respect to their composition: (I) cellular TM and (II) soluble TM (sTM), a proteolitically cleaved fragment of cellular TM which shows main properties of cellular TM [8]. Cellular TM consists of three portions – the extramembrane, transmembrane and intracellular. The extramembrane portion consists of three domains: N-terminal lecitin-like domain (D1) in which antiinflammatory properties of TM are contained, followed by EGF-like domain (D2) that can bind thrombin, and an O-glycosylation-rich domain (D3) [9].

sTM exists in plasma and urine [10], and in amniotic fluid [unpublished data]. It is often used as a marker of endothelial injury in various clinical settings: in disseminated intravascular coagulation (DIC), myocardial infarction, venous thromboembolism and others [8,11]. A significant increase of sTM was observed in preeclampsia [12] and pregnancy induced hyperten-

sion (PIH) [13], as well as in acute placental abruption [14], but not in recurrent pregnancy loss [15].

It has been suggested that TM is a key component in control of the fluidity of blood in the intervillous space and therefore protects the utero-placental circulation against local thrombosis (placental thrombosis) [16]. In our working hypothesis we assume that not only TM from the placenta, but also TM from myometrium, a highly vascularized tissue, can induce locally APC production and thus prevent the utero-placental circulation from hypercoagulability as well. To determine whether this is potentially possible, we decided to measure TM concentration in placenta and myometrium, and also in fetal membranes.

Material and methods

Patients

The study group consisted of 35 parturient women (23.8±3.1 of age), 21 primapares and 14 multipares, with a normal course of pregnancy (we excluded from our analysis complicated pregnancy, such as placenta previa and low-lying placenta, as well as placental abruption, preeclampsia, prolonged rupture of membranes and intraamniotic infection). Indications for cesarean section were as follows: fetal distress in a subgroup of labouring patients – 7; a subgroup of elective sections: patients with two cesarean births in anamnesis – 4, with more than three cesarean births – 3; breech presentation with a primapara – 5; breech presentation with a patient after a cesarean section – 4; transversal position – 2.

The control group consisted of 20 healthy, nonpregnant women, 20-25 years old, in the luteal phase of menstrual cycle.

All women were informed about the research and they accepted the sampling of placenta, myometrium, and blood. Permission of the Bioethics Committee was also obtained.

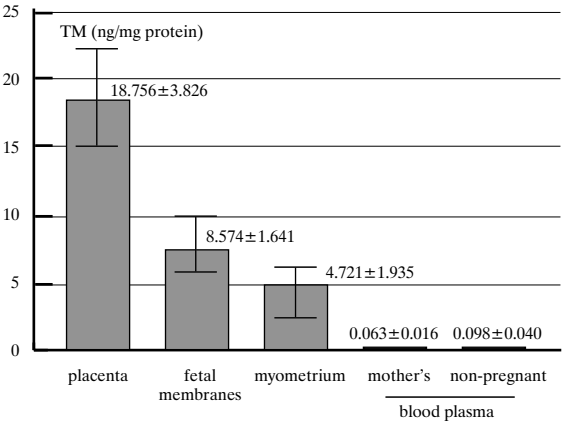
Sampling of the material

All materials were obtained at the time of the caesarean section: (I) strips of myometrium of 3.0-5.0 g from the lower segment of the uterus (the decidua was cut off and disposed of); (II) strips of placenta of ca 5.0-10.0 g from the central part of its maternal side; (III) strips of fetal membranes of ca 3.0 g from the distal part. The strips were placed in hermetic test tubes after rinsing with 0.9% NaCl and stored for 3-6 weeks in -70°C; (IV) blood samples were obtained from antecubital veins without venous occlusion. The anticoagulant/blood proportion was 1:9 (one part of 3.2% sodium citrate, nine parts of the blood). The blood was placed in a plastic test-tube which was put in icy water and taken to the laboratory to be centrifuged (2500 x g, 20 min, +4°C). The blood plasma was divided into 200 µl portions in test tubes which were closed tightly and stored for 3-6 weeks at –70°C.

Preparation of the tissue extracts

To prepare tissue extracts we followed the procedure recommended by American Diagnostica Inc. In short, frozen tissue strips were pulverized within liquid nitrogen, then detergent extraction was applied (10% Triton X – 100 in Tris buffer, pH 8.5, 12 h at +4°C). The obtained suspended matter was centrifuged at 100 000 g for 60 min at +4°C. The supernatant was divided into 200 µl portions, placed in tightly closed plastic test-tubes, and kept for 1-2 weeks at -70°C.

Figure 1. **Concentration of cellular thrombomodulin (ng/mg of total protein) in placenta, fetal membranes and gestational myometrium, as well as soluble thrombomodulin in blood plasma of parturient and nonpregnant women**



Laboratory measurements

The concentration of thrombomodulin was measured by an immunoenzymatic method (ELISA). IMUBIND Thrombomodulin ELISA Kit by American Diagnostica GmbH was used. The manufacturer's instructions were strictly followed. The samples were assayed in batch operations. Total protein concentration was measured by BCA method using bicinchoninic acid. TM concentration was expressed in ng/mg of total protein. The interassay and intraassay coefficients of variability were less than 10%.

Statistical analysis

The results are presented as mean values with standard deviations (x±SD). Statistical analysis was done with Microsoft® Excel 2000 and Statistica for Windows 5.0 by StatSoft®. t-Student test was used for analysis of measurements in gestational tissues and mother's blood (dependent groups). Data between mother's blood and the blood of nonpregnant women (control) were analysed by unpared test of Mann-Whitney. Pearson's method was used for correlation analysis. The value $p<0.05$ was taken as statistically significant.

Results

1. Cellular TM in gestational tissues (placenta, fetal membranes and myometrium) (n=35):
Concentration of TM antigen in placenta was 18.756± ±3.826 ng/mg proteins, in fetal membranes 8.574±1.641 ng/mg proteins, and in the myometrium 4.721±1.935 ng/mg proteins. The differences between the particular values were highly significant ($p<0.0001$) (*Fig. 1*).

2. Soluble TM in mother's blood (n=35) and the blood of nonpregnant women (control) (n=20):

Concentration of TM antigen in the mother's blood plasma was 0.063±0.016 ng/mg proteins, and in blood plasma of nonpregnant women 0.098±0.040 ng/mg proteins. The difference was not significant statistically ($p>0.05$).

3. Comparison of TM concentration in blood and tissue extracts:

The ratios between soluble TM in blood plasma and cellular TM in tissue extracts were as follows: plasma/placenta – 1/298.4; plasma/fetal membranes – 1/136.5; plasma/myometrium – 1/74.6.

4. Correlation analysis:

No correlation was found between TM concentrations in plasma and placenta extract ($r=-0.1709$, $p=0.326$), blood plasma and fetal membranes extract ($r=-0.055$; $p=0.754$), as well as between concentrations in plasma and myometrium extract ($r=-0.0927$, $p=0.596$).

Discussion

A number of authors [2,17,18] have studied TM in placenta, but not yet in gestational myometrium and fetal membranes. In fact, we have found only one mention of TM measurements in 'uterus/ovary' of mice without information whether gestational myometrium was examined [19]. In our study we found out that TM is also present in gestational myometrium and fetal membranes, although in concentration lower than in the placenta. Originally TM was isolated from human placenta by Salem et al. in 1984 [17].

In placenta, TM was immunolocalized to syncytiotrophoblast and fetal vascular endothelium [2]. As concerns the localisation of TM in myometrium, we contemplate two options: either (I) TM comes from abundant vascularisation of gravid uterus, or/and (II) from the cytotrophoblast which invades spiral arteries of myometrium in the course of pregnancy [20]. We cannot exclude that TM measured by us in extract of fetal membranes might come from the fragments of decidua, which are broken off during labour. Anyway, immunohistochemical studies are needed to answer the question about localisation of TM in myometrium and fetal membranes.

Many hemostatic and nonhemostatic functions are ascriebed to placental TM.

TM is considered to be a major determinant of the antithrombogenity of placental bed via protein C anticoagulant pathway that controls the balance between coagulation and anticoagulation/fibrinolys [16,21]. As epithelial protein C receptors (EPCR) are also localized in syncytiotrophoblast [5], it would imply that TM activity in the intervillous space can be even amplified. Moniva [7] holds that APC is the main anticoagulant of intervillous space and shows that APC can release urokinase plasminogen activator (uPA) from uPA/PAI-1 complex enhancing in this way the protective fibrinolysis. In our working hypothesis we assume that myometrial TM can play a role similar to that of placental TM. The anatomic and functional integrity of uterine and placental segments of the utero-placental circulation might be a supplementary argument.

As regards to the clinical implications of our results, we assume that placenta and myometrium, when destroyed by retroplacental hematoma at placental abruption, can release TM into mother's blood and that would be the explanation for increased plasma level of TM in this complication which was observed by other authors [14].

Furthermore, cellular TM might be critical in reproduction, mainly in implantation and placentation as well as in early post-implantation embriogenesis. In animal experiments (mice), a deficit of TM or EPRC turned out to be a lethal factor [22]. Isermann et al. [23] have suggested that the thrombomodulin-protein C system is essential for maintaining pregnancy. Two distinct mechanisms were considered in intrauterine fetal growth retardation (IUGR): one of them is the result of engagement of protease-activated receptors (PAR's), PAR-2 and PAR-4 or both, by thrombomodulin-protein C system, and the second is TM-independent cytotoxic effect by fibrin degradation products on cytotrophoblast.

Taking into account and consideration our earlier studies on plasminogen activators and plasminogen activator inhibitors (PAs/PAIs system), and receptors for urokinase (uPAR) as well as tissue factor and tissue factor pathway inhibitor (TF/TFPI) in gestational myometrium [21,24,25], we would like to imply that gestational myometrium is parallely to placenta a source of many components that are active in coagulation and fibrinolysis. The presented results support the hypothesis that not only placenta but also myometrium can be the source of tissue TM, and that both, placental and myometrial TM can be of importance for intervillous blood fluidity.

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Oxidative stress in burnt children

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Abstract

Background: One of the effects of burn injury is production of reactive oxygen species increasing general-structural damage. Such a condition is called oxidative stress. The purpose of this research was to find out whether oxidative stress is present in burnt children treated routinely and, if so, in which phase of the disease it is the most severe and how long it persists.

Material and methods: The study was carried out on a group of 84 burnt children. The patients were divided into 2 groups: lightly burnt (LB-N:55) and moderately to severely burnt (SB-N:29). Blood samples were collected based on hospitalization period within the 1st, 2nd, 3rd, 7th and 21st day, respectively, following the injury. Total antioxidative capacity (TAC) in plasma and concentration of lipid peroxidation products (TBARS) in red blood cells were estimated. The test results were compared to control group of 40 healthy children.

Results: The research showed a statistically significant decrease in TAC in both groups of burnt children. The TBARS concentration was increased in both groups within the 1st day following burn injury and maintained the high level throughout the research continuation. No statistically significant differences between LB and SB groups were recognized.

Conclusions: The observed changes in the tested parameters are attributable to oxidative stress occurring in burn disease. For this reason, burn – injured children are recommended to receive exogenous antioxidants.

Key words: children, burns, oxidative stress, lipid peroxidation, antioxidants.

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Introduction

Among various pathophysiological mechanisms in burn disease, the subject of reactive oxygen species (ROS) has been drawing interest since the early 70's. The ROS are mostly free radicals including such molecules as: superoxide radical anion, hydroperoxyl radical, singlet oxygen and hydrogen peroxide. The results of previous research show that burn injury is followed by ROS generation due to hypoxia – reperfusion (dehydrogenase change into xanthine oxydase in vascular endothelium cells) and activation of immune system (NADPH oxydase reaction in phagocytic cells). The excessive increase in ROS level can lead to oxidative stress defined as prooxidant – antioxidant equilibrium disturbance [1,2].

The most frequently used marker of oxidative stress is a measurement of concentration of lipid peroxidation products including lipid peroxides, aldehydes (malonyldialdehyde MDA), alkenales. The lipid peroxidation results from ROS attacking mainly the cell membranes, which leads to an attenuation of membrane flexibility, disturbance of asymetric distribution of membrane phospholipides, elevation of permeability for hydrogen ions, dysfunction of Na^+K^+ and $Ca^{2+}Mg^{2+}$ ATPases, etc. [3,4].

Due to adverse events of ROS generation, cells are equipped with protective mechanisms against oxidative stress. It is an antioxidative defence involving low-molecular compounds (α-tocopherol, ascorbic acid, β-carotene, glutathione, Q-coenzyme, uric acid, bilirubin, etc.), enzymatic compounds (superoxide dismutase SOD, catalase CAT, glutathione peroxidase GPx, etc.), proteins transporting and storing ions of metals (ferritin, transferrin, ceruloplasmin, metalothionein). Most of the mentioned antioxidants affecting total antioxidant capacity (TAC) are found in plasma. The TAC means an ability to scavenge ROS and prevent lipid peroxidation. The marker was introduced by Ingold and Burtin as an antioxidative potential in subjects. The TAC involves mainly includes uric acid, ascorbic acid, α-tocopherol and albumin [5,6].

The world literature of medicine provides with little infor-

mation on the role reactive oxygen species play in burn pathogenesis in humans, particularly, children. Attempts to diminish burn effects, namely, oxidative stress by antioxidants including α-tocopherol, superoxide dismutase have been made, however, without consistent results [7-10].

The purpose of the research was to find out:

– whether oxidative stress occurs in burnt children treated according to methods of burn treatment,

– in which phase of the disease the severity and persistence of oxidative stress is the greater

– whether treatment requires adjusting intended to prevent or attenuate harmful effects of reactive oxygen species reaction on a patients.

Material and methods

Subjects

The research test was carried out on a group of 84 burnt children admitted to the pediatric surgery ward in County Hospital in Gorzów Wlkp. from 1998 to 2000. The children's age ranged from 5 months to 14 years old. The patients were divided into 2 groups:

– LB – lightly burnt – first and second – degree burns, less than 10 percent of the total body area (TBA) (55 children)

– SB – moderately to severely burnt (second – degree burn, more than 10 percent of the TBA to third – degree burn requiring over 7 days of hospitalization (29 children).

Average duration of hospitalization for slightly – burnt children and moderately to severely burnt ones was 3.5 days and 16.5 days, respectively.

The test covered only those children who were admitted to hospital within the first day following burn injury. The patients were treated routinely by methods accepted in the general and local burn treatment of children. Fluid resuscitation was carried with the Parkland method. It was being adjusted based on clinical evaluation of the patient's general condition, aiming at reaching minimal diuresis 1 ml/kg per hour. The intravenous co-administration of calcium preparations and vitamin C was initiated within the first day following burn injury. In addition, daily treatment with 20% albumin solution at 2-3 g/kg b.m. was implemented in the patients within the 36th-48th hour. Morphine i.v. and paracetamol per rectum were applied as analgesics. Based on a degree of burn injury antibiotic therapy was begun either within the first day or later adequately to an antibiogram received from the burn wound swab. The children were fed orally or through the nasogastric tube soon after they had recovered from the trauma. Parenteral nutrition following the procedure by Sporadyk and Puchała was applied [11,12]. In the burn injuries requiring surgical treatment an early necrectomy was performed within the 3rd to 5th day after burn injury usually simultaneously with the skin grafting. In the burn injuries of varied severity or particular anatomic location (fingers and intradigital spaces) dermabrasion was applied. In total, there were 27 such treatments performed in 16 patients. None of them required escharotomy.

Vein blood samples were collected from both groups on the 1st and the 3rd day after burn injury and, additionally, from the group of moderately to severely burnt on the 7th, 14th and 21st day, responsively, appropriate for duration of hospitalization. The blood samples were always collected together with samples for routine laboratory tests. The test results in the injured children were compared with the control group of 40 healthy children admitted to hospital for planned surgical treatment.

The assembly has got an approval of Bioethical Committee at District Chamber of Physicians in Gorzów Wlkp. to carry out the research. In case of each burn-injured child and the child from control group a written permission was given by their parents to use the blood samples in order to estimate oxidative stress markers.

Biochemical analysis

The vein blood samples for estimation of oxidative stress parameters were collected into polyethylene tubes containing potassium EDTA. The blood was centrifuged (750 g, 4°C, 10 min), plasma was removed and stored at -20°C until analysis. Erythrocyte fractions were resuspended and washed three times with cold isotonic saline solution. Washed erythrocytes were stored at -20°C until analysis (up to seven days).

In the erythrocytes lipid peroxidation was estimated using the measurement of thiobarbituric acid – reactive substance (TBARS) level according to Buege and Aust method [13].

In the plasma total antioxidant capacity (TAC) was determined using a diagnostic kit Randox (UK).

Hemoglobin was estimated with Drabkin standard method.

Statistical analysis

All data are presented as mean ± SD and analyzed by "Statistica for Windows" program, using U Mann-Whitney test and a model of multidimensional regression. The accepted level of significance was $p<0.05$.

Results

The obtained test results are presented in *Tab. 1,2* and *Fig. 1,2*.

The concentration of lipid peroxidation products (TBARS) was statistically significantly higher in the groups of lightly burnt children (LB) and moderately to severely burnt (SB) in comparison with the control group. The highest average values of TBARS concentration were observed in SB group within the 21st day of the examination. Analyzing the diagram of distribution of the TBARS concentration values according to the test duration (*Fig. 1*), a constant and uniform increase in TBARS concentration was observed in both groups of burnt children throughout the whole period of the examination. No statistically significant changes between LB and SB groups were observed.

Total antioxidant capacity (TAC) was significantly lower in groups of lightly burnt (LB) and moderately to severely burnt (SB) children in comparison with the control group. The lowest average TAC values were observed in LB and SB groups within 3rd and 7th day, respectively, following burn injury. Distribution of TAC concentration according to test duration is illustrated in the *Fig. 2*. It indicates that the concentration of antioxidants was lower in LB group compared to control group on the

Table 1. **Concentration of lipid peroxidation products (TBARS) in subjected groups (mean±SD)**

	Control	LB-1	LB-3	SB-1	SB-3	SB-7	SB-14	SB-21
N	40	55	23	29	29	29	13	7
TBARS μmol/gHb	3.03±0.65	5.66±1.56*	5.33±1.35*	5.67±1.75*	5.63±1.58*	5.88±1.28*	5.98±0.88*	6.57±1.25*

Table 2. **Total antioxidant capacity (TAC) in subjected groups (mean±SD)**

	Control	LO-1	LO-3	CO-1	CO-3	CO-7	CO-14	CO-21
N	40	55	23	29	29	29	13	7
TAS mmol/l	1.34±0.20	1.28±0.28	1.05±0.20*	1.19±0.20*	1.11±0.16*	1.06±0.17*	1.18±0.22*	1.13±0.26*

* Statistically significant differences ($p<0.05$) in relation to control group

Figure 1. **Distribution of lipid peroxidation products (TBARS) in accordance with examination time**

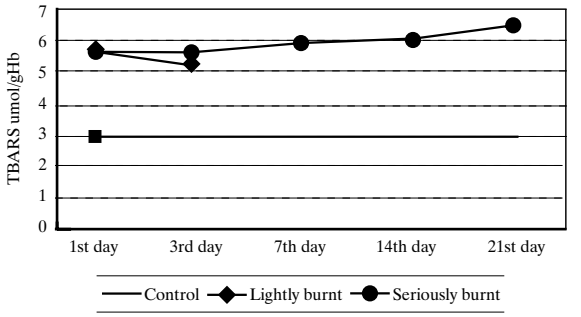
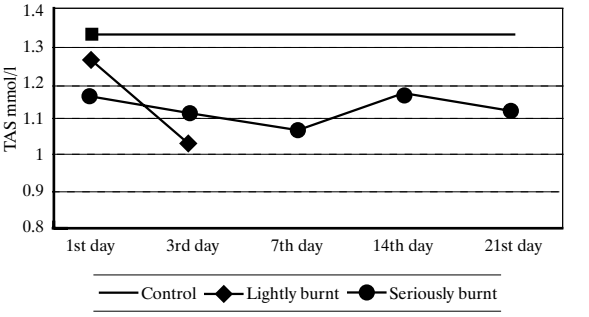


Figure 2. **Distribution of total antioxidant capacity (TAC) in accordance with examination time**



1st day following injury ($p>0.05$) and on the 3rd day of the clinical trial there was a further decrease in TAC, which was significant statistically comparing with control group. In SB group a statistically significant decrease of the TAC was observed from the very 1st day ($p<0.05$), which maintained until the research was completed. No statistically significant differences between LB and SB groups were noted on particular days of the research.

Discussion

A thermal injury is followed by a significant increase in generation of ROS in the body. Within the early phase of the disease ROS generation occurs in hypoxia and damaged capillaries in the injured area. Such a condition causes the xanthine dehydrogenase to convert into xanthine oxydase using molecular oxygen as an electron acceptor after reperfusion. Its result is formation of superoxide radical anion [14,15]. In further phases of the disease ROS level may be similar to the initial one, but their source is different. Injury-activated neutrophils migrate not only to the injured skin area but also to remote organs, especially lungs. The activated neutrophils are considered to be the main source of ROS generation in further phases of burn disease [7,16]. Another source of ROS is the release of ferric and copper ions from the damaged cells inducing an increase in hydroxyl radical production by Fenton reaction. The role of the released ferric and copper ions in ROS generation is confirmed by tests proving that ferric chelator (deferoxamine) administration in the post-burn resuscitation decreased significantly a demand for fluids and diminished the red cells hemolysis [17,18].

Analyzing the burn injury influence on concentration of lipid peroxidation products (TBARS) in red cells in burnt children, their statistically significant elevated level was recognized in all the tested groups. An increase in TBARS concentration appeared as early as on the 1st day following burn injury. The finding is consistent with most observations apart from Guerbez [19] who did not confirm any increase in products reacting with thiobarbituric acid in lung tissue, liver or stomach of burnt rats. Neither did the author recognize any increase in protein peroxidation level. In other studies there was observed enhanced peroxidation of lipids in burnt skin and lungs, with their highest values appearing 15 min and 2 h after burn injury and, respectively, after 30 min and 30 h, in plasma [20]. Besides, the observations confirmed a markedly elevated level of lipid peroxides in the skin and plasma 1 h after burn injury, reaching 6-times higher level 3 h following the injury compared to control group [21]. Other tests showed an increased level of lipid peroxidation products in the plasma and in lungs and liver within the 4th day after burn [22,23]. An increase in conjugated dienes, lipid peroxides and malonyldialdehyde contents in plasma and lung tissue was observed in animals 2 h after burn injury [24]. In an experimentally produced multiorgan failure syndrome (MOF) in rats, there was recognized an increase in TBARS within the 1st and 2nd day [25]. The findings are in agreement with clinical test in burnt patients, expressing an increase in lipid peroxidation products concentration as early as within the 8th h, the 1st and 2nd day after burn injury [26-28].

Lipid peroxidation was strongly expressed, which was confirmed by the persistence of elevated TBARS concentration in the burn disease (in the present studies – till the 21st day after burn). The results obtained by other authors were consistent. However, it should be noted that most of the experimental tests were carried out within short time periods. Szpringer et al. [24] were observing an increase in plasma lipid peroxidation products concentration within the 48 h, Dargani et al. [29] – in the lungs and liver within the 3rd day, Demling and Lalonde [30] in the plasma within the 5th day after burn injury. Nishigaki et al. [21] recognized an elevated level of lipid peroxidation products in the burnt skin and plasma maintaining till the 7th day, since which it returned to control group values (the tests were carried out till the 28th day). Van Bebber et al. [31] doing experimental tests on MOF observed an increased level of the TBARS until the 14th day. The results of clinical observations show high concentration of lipid peroxidation products maintaining until completion of the tests: Hongming et al. [26] – till the 3rd day, Wooliscroft et al. [27] – till the 5th day and Kumar et al. [28] – till the 10th day, respectively, after the injury.

The increase in concentration of TBARS is likely to be related to the specificity of injury, not its severity. It is confirmed by the lack of statistically significant differences in TBARS concentrations between LB (first- and second-degree burn) and SB (second- to third-degree burns) groups. Besides, the fact is stated by other authors who observed no correlation between malonyldialdehyde concentration and the area of burn [28]. Moreover, the levels of lipid peroxidation products were comparable in the patients with either severe or light burn [9]. In the tests by Gosling et al. [32] concentration of lipid peroxidation products was higher in more severely burnt patients, but had no correlation with the patients' death rate though. Madsuda et al. [33] found out that changes in antioxidants concentration and the level of oxidative damage in the plasma appear only with deeper skin layer injury.

There are much fewer studies concerning evaluation of total antioxidant capacity (TAC) of plasma than those describing changes in concentration of lipid peroxidation products. Our results show that TAC level was decreased in both groups of burnt children. In the group of moderately to severely burnt, total antioxidant status of the plasma did not return to control values until the 21st day following burn. Demling et al. [34] presented their test results showing that the decrease in antioxidants concentration and the increase in lipid peroxidation with inhale burn injury. Cetinkale et al. [22] observed TAC decrease 24 h after burn injury. The analysis of particular elements of total antioxidant status in burn blisters suggested that protein concentration was by 54% lower in bluster fluids than in plasma and concentration of bilirubin by 32% respectively. However, uric acid level was unchanged in plasma [35]. Other clinical tests showed a significant decrease in plasma α-tocopherol, thiols and increase in conjugated dienes levels in burnt patients [36].

Few studies confirmed the correlation of oxidative stress indicators with the patients' clinical condition and the burn disease course [32-34]. However, in our tests no correlation between the children's clinical condition and the changes in lipid peroxidation products concentration was recognized. The TBARS and TAC concentrations were the same both in the group of lightly burnt and moderately to severely burnt children. The lightly burnt children were discharged from hospital in good clinical condition after 3.5 days of hospitalization. In LB group the levels of the tested parameters on the 3rd day were not statistically different from the levels obtained in SB group hospitalized for 16.9 days or the patients requiring surgical treatment. It is worth noticing that values of TBARS and TAS concentrations at the beginning of the burn disease did not differ from the results obtained on the 21st day in the severely burnt children during recovery phase.

Total antioxidant status (TAC) in the plasma involving the activity of all the compounds being able to play a role of free radicals scavengers (uric acid, vitamin C, E, bilirubin, albumin) appears to be a very sensitive marker of the oxidative stress. The decreased TAC together with an elevated level of lipid peroxidation products persistent through the burn disease duration is attributable to ROS generation and oxidative stress. Immunological defence mechanisms in burnt children are disturbed not only due to the damaged skin – an important protective barrier, but also repeated surgical treatments under general anaesthesia, antibiotics, ointments and other therapy applied locally on the burn wound. Besides, the increased catabolism, a nitric imbalance and other metabolic irregularities make the contribution to oxidative stress. The described changes in the oxidative stress markers are likely to play a role in the pathophysiology of immunosuppression present in burnt patients and response of the body to so-called "second hit" (f.e. sepsis) affecting the development of MODS [37,38].

Conclusions

Burn injury induced oxidative stress in the examined groups expressed as an increase in concentration of lipid peroxidation products (TBARS) in red blood cells and a decrease in total antioxidant capacity (TAC) in plasma. The process started immediately after the injury and persisted through the whole course of study (till the 21st day after burn).

The observed irregularities were caused by the specificity of injury regardless of its severity and the described changes were comparable in both groups of burnt children.

An attenuation of total antioxidant capacity (TAC) in plasma and enhancement of lipid peroxidation (TBARS) in erythrocytes maintaining during the study may be related to immunosuppression present in burnt children and their high sensitivity to so-called "second hit". For the reason, burn-injured children are recommended to continue long-term supportive therapy wit exogenous antioxidants.

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Gastroesophageal reflux (GER) in children and adolescents with regard to food intolerance

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Abstract

Purpose: The hypothesis put forward in the current study was that food intolerance can be a cause of gastroesophageal reflux (GER) affecting children with this pathology at various age. In order to confirm or exclude this hypothesis, the study objective was to determine the frequency of the cause-and-effect relationship between allergy to cow milk proteins and/or other food products and gastroesophageal reflux found in the study group of patients, and to establish diagnostic differentiation guidelines in GER caused by food intolerance, i.e. secondary vs primary GER.

Material and methods: A total of 735 children (428 boys – 58.2% and 307 girls – 41.8%; mean age x=41.12 months ±25.03) with symptoms suggesting gastroesophageal reflux disease (GERD) were qualified for the study.

The diagnostic procedure included a 24 h pH-metry of the oesophagus, which was perfomed in all the study children. In 703 patients (411 boys – 55.9% and 292 girls – 39.7%) upper gastrointestinal endoscopy was carried out. Manometric examination of the oesophagus was performed in 232 children (123 boys – 16.7% and 109 girls – 14.8%). Allergological and immunological tests were done in 170 children with suspected allergy (91 boys – 12.4% and 79 girls – 10.7%). Contrast radiography of the upper gastrointestinal tract was performed in 78 children with respiratory symptoms (42 boys – 5.7% and 36 girls – 4.9%). Oral challenge test was used to differentiate primary GER from GER secondary to cow milk proteins intolerance or other food allergy in 138 children (72 boys – 9.8% and 66 girls – 8.9%).

Results: Based on the 24 h pH-metry of the oesophagus and endoscopic examination of the upper gastrointestinal tract, gastroesophageal reflux disease and/or reflux oesophagitis were diagnosed in 138 study subjects (18.8%); mean age x=23.36 months ±22.53. Positive oral food challenge test confirmed pathological GER secondary to cow milk protein allergy/other food hypersensitivity in 62 children (8.4%).

Conclusion: The current study revealed the existence of the cause-and-effect relationship between allergy to cow milk protein/ /other food products and GER in the study children at various age.

Key words: acid gastroesophageal reflux: primary, secondary; food allergy, oral food challenge test, children.

Introduction

Gastroesophageal reflux (GER) occurs when stomach contents leak back involuntarily into the oesophagus due to functional insufficiency of certain elements of the antireflux barrier, particularly due to lower oesophageal sphincter (LES) dysfunction [1-4].

Pathogenetically, gastroesophageal reflux has been divided into primary and secondary [1,2,5-8]. Primary gastroesophageal reflux (idiopathic), depending on its intensity, can be physiological or pathological having typical or atypical manifestation.

Secondary gastroesophageal reflux is usually a pathological condition observed in such diseases as infections, allergies, as well as systemic, genetic, metabolic disorders and others [2,9,10].

Reflux of varied intensity may occur at any age, but in children it is intensified in the first months or years of life [2,4].

In approximately 40-50% of infants, GER is functional in nature, uncomplicated and regarded as physiological [11-13]. In this subgroup, in approximately 60-70% of children reflux symptoms subside spontaneously (usually between 10th-12th month of life); in 25%, the symptoms persist for over a year, while in the rest (ca. 5%) even longer (18th-24th month of life) [1,10-14].

In approximately 30% of children, shift of stomach contents into the oesophagus is so intensified that reflux becomes a pa-

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Table 1. **Children treated in the Department of Children Diseases in Białystok, in the years 1992-1995 r., qualified for the study and subjected to clinical observation**

Hospitalization and treatment of patients in the Department – study period (in years)	Children hospitalized due to various ailments	Children with suspected gastroesophageal reflux disease (GERD)		Children with suspected GERD and positive family history of alimentary disorders		Children with suspected GERD and negative family history of alimentary disorders	
	Number	Number	[%]	Number	[%]	Number	[%]
1992/1993	2872	278	9.7	144	5.0	134	4.7
1994	2259	203	9.0	57	2.5	144	6.5
1995	2722	254	9.3	63	2.3	191	7.0
Total	7853	735	9.4	264	3.4	471	6.0

thology (primary or secondary) and usually causes troublesome symptoms from the oesophagus (typical), frequently enhanced by mucosal inflammation [1,10-15].

Reflux can also be responsible for diverse atypical complaints – respiratory, circulatory, neurological or for all-systemic manifestation [2,4,10]. Duration of reflux symptoms is changeable, as they may become chronic or recurrent.

The presence of clinical symptoms of GER can be associated with the co-occurence of food allergy, especially cow milk protein intolerance (mainly in infancy) or other food products popular with children in the later period (secondary reflux) [6,9,10].

In the earliest developmental period, reflux may be caused by food allergy alone, at the later age, food intolerance may co-occur with GER [6-8,16-19].

Diagnostically and therapeutically, differentiation between primary (idiopathic) and secondary (related to food allergy) GER is essential.

The observations and clinical findings obtained in the last 10 years in the III Department of Children's Diseases in Białystok as well as the data reported by many researchers seem to confirm that there is a cause-and-effect relationship between gastrointestinal reflux and food allergy, mainly cow milk protein intolerance in infancy, and allergy to other food products at the older age [6-8,18,20,21].

Research hypothesis – food allergy can lead to acid gastroesophageal reflux in children at various age.

In order to confirm or exclude this hypothesis, the following study objective was formulated: 1) to determine the frequency of cause-and-effect relationship between allergy to cow milk proteins and/or other food products and acid gastroesophageal reflux found in the study group of patients; 2) to establish rules describing diagnostic differentiation guidelines in acid GER caused by food intolerance, i.e. secondary vs primary GER.

Material and methods

A. Patients' profiles

Within a 3-year period, i.e. in the years 1992-1995, 7853 children were hospitalized in the III Department of Children's Diseases, Medical University of Białystok (*Tab. 1*, *Fig. 1*).

Of them, 735 (9.4%) children were selected, with varied mono- and poly-organ symptoms suggesting gastroesophageal reflux disease (GERD).

According to age of the study children, the following symptoms caused by massive shift of the stomach contents back to the oesophagus were distinguished:

a) from the alimentary tract – intensive regurgitation, vomiting, irritability/sudden crying, ruminatio, food refusal/feeding disturbancies, failure to thrive, abdominal pain, heartburn, belching/hiccups, bad breath (foetor ex ore), swallowing difficulty (dysphagia);

b) recurrent or chronic respiratory disorders – apnoea/desaturation, frequent pharyngitis/tonsilitis, bronchitis, obturative/spastic bronchitis, pneumonia, persistent cough, wheezing;

c) neurological symptoms suggesting GERD – back arching, flaccidity, pallor, disorders of consciousness, stiff neck (torticollis);

d) and others – e.g. anaemia.

B. Diagnostic investigations

General characteristics of diagnostic investigations performed in 735 (100.0%) children suspected of GER has been presented in *Tab. 2*.

The study group consisted of 428 boys (58.2%) and 307 girls (41.8%), aged 1.5-168 months; mean age x=41.12 months (3 and 5/12 years) ±25.03.

Diagnostic procedures included 24 h pH-metric testing of the oesophagus done in all the 735 study children. The following pH monitoring parameters were analysed: the number of episodes of acid GER (a decrease of intraesophageal pH below 4.0), the number of episodes of acid GER lasting more than 5 minutes (so-called "long episodes"), reflux index (total and supine – in both leads) that measures the percentage of time that the pH is below 4.0 within 24-hour intraesophageal pH monitoring [6,7,14,17,22-29]. Endoscopic examination of the upper gastrointestinal tract performed in 703 patients (411 boys – 58.5% and 292 girls – 41.5%, aged 4 – 168 months; mean age x=43.20 months (3 and 7/12) ±26.74) [8,15,26,27,29,30].

A total of 232 (31.5%) children (123 boys – 16.7% and 109 girls – 14.8%, aged 4-102 months; mean age x=25.42 months ±21.47) with suspected GER underwent oesophageal manometric investigations at the time of final diagnosis to assess motoric function of the oesophagus, especially of its lower sphincter (LES) [26,27,31]. Allergological and immunological tests were performed in 170 (23.1%) children (91 boys – 12.4% and 79 girls – 10.7%), mean age x=23.47 months ±19.23 to search for allergic cause of GER [19,32].

***Figure 1*. Qualification of hospitalized children with symptoms suggesting gastroesphageal reflux disease (GERD). Diagnostic examinations confirming or excluding gastroesphageal reflux (GER)**

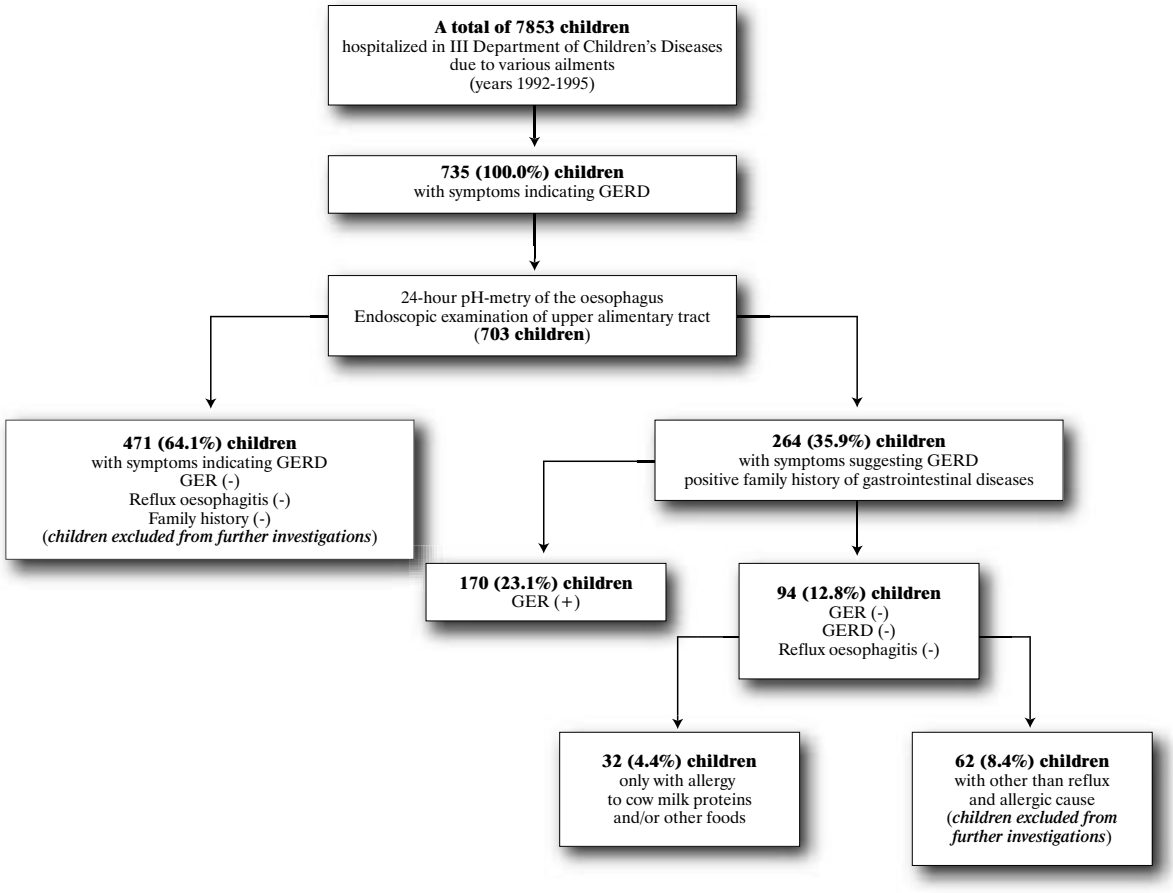


***Table 2*. Diagnostic investigations in 735 children with suspected gastroesophageal reflux disease (GERD)**

Preliminary diagnostic investigation	Study children			Age of study children from – to Mean age [x]
	Gender	N	%	
24-hour oesophageal pH-metry	boys	428	58.2	> 1.5-168 months (41.12 +/- 25.03)
	girls	307	41.8	
	total	735	100.0	
Endoscopic examination of oesophagus, stomach and duodenum	boys	411	55.9	>4-168 months (43.20 +/- 26.74)
	girls	292	39.7	
	total	703	95.6	
Oesophageal manometry	boys	91	12.4	>4-102 months (23.47 +/- 19.23)
	girls	79	10.7	
	total	170	23.1	
Allergological and immunological investigations	boys	123	16.7	>4-102 months (25.42 +/- 21.47)
	girls	109	14.8	
	total	232	31.5	
Radiological examination of upper alimentary tract with barium contrast	boys	42	5.7	>4-102 months (23.36 +/- 22.53)
	girls	36	4.9	
	total	78	10.6	

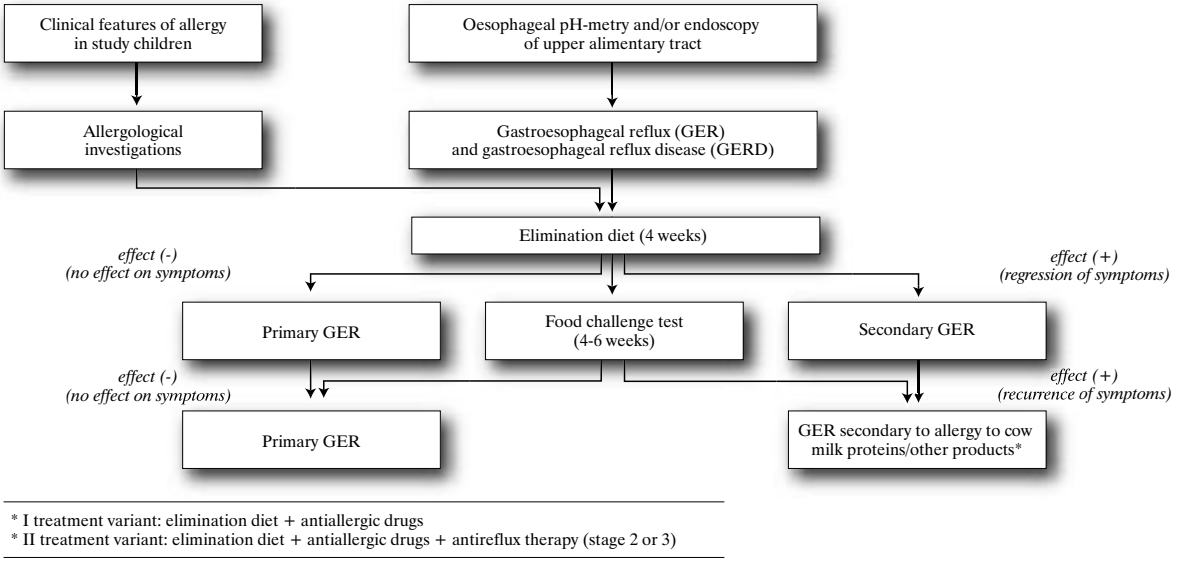
The upper gastrointestinal contrast X-ray examination was done in 78 (10.6%) children with respiratory symptoms (42 boys – 5.7% and 36 girls – 4.9%; mean age x=23.26 months ±22.53, to look for evidence of the causative role of GER in their development, and to search for likely developmental anomalies frequently associated with defects of the trachea, the bronchi or the diaphragm [27,28,33,34].

Searching for a cause of GER other than food allergy, the respective findings of accessory investigations were analysed.

Chest X-ray pictures and computer tomography of nasal sinuses were used to evaluate the type, location and character of the associated pathology.

In order to confirm or exlude the infectious cause of GER, the following parameters were estimated: erythrocyte sedimen-

Figure 2. **Algorythm of management differentiating between primary and secondary GER in 138 study children with suspected allergy to cow milk proteins and/or other foods based on elimination diet and challenge test outcomes**



* I treatment variant: elimination diet + antiallergic drugs
* II treatment variant: elimination diet + antiallergic drugs + antireflux therapy (stage 2 or 3)

tation rate (ESR), C-reactive protein (CRP), antistreptolysin reaction (ASR), blood smear morphology, proteinogram, protein immunoelectrophoresis, immunoglobulins A, M, G, and iron concentration.

Bacteriological analysis involved testing of blood, urine, faeces, bile, as well as pharyngeal and nasal swabbings in some children.

Metabolic screening of blood (e.g. lactate acid, ammonium, acid-base equilibrium parameters) was performed to exclude the metabolic cause of the disease, while pilocarpin test (sweat chloride) was used to exclude mucoviscidosis in these children [2,4,9,10].

We used our own algorythm of diagnostic and therapeutic management to differentiate primary (idiopathic) from secondary GER (*Fig. 2*) in 138 children with gastroesophageal reflux disease (GERD) out of 170 with acid GER [8].

As food allergy was believed to trigger or enhance GER in the study group of children, oral challenge test was conducted to determine its allergic background [6,7,18,20,32].

At the first stage, elimination diet was introduced for 4 weeks to exclude food products suspected of allergisation, including cow milk or others (depending on the child's age).

Instead, the youngest patients received milk substitutes – casein hydrolysates, whey proteins or a mixture of free amino acids. Older children had hypoallergic diet without harmful products [10,16,32].

The patients who showed clinical improvement (regression or alleviation of symptoms) were subjected to oral food challenge with a milk mixture, diary products or other harmful foods which they had consumed before (open method) [32].

The diagnosis of allergy to cow milk protein and/or other foods was made when the symptoms subsided during the application of elimination diet and the food challenge was positive (the symptoms recurred).

Intensity of reflux symptoms, especially those typical (from the alimentary tract), was assessed using a "stipulated" score system (1-5):

– mild short-term symptoms (up to 2 weeks), occurring episodically or recurring periodically – 1,
– chronic symptoms (over 2 weeks), mild in intensity – 2,
– chronic symptoms of considerable intensity – 3,
– chronic symptoms of considerable intensity, with a periodical tendency to exacerbate – 4,
– chronic symptoms of high severity – 5.

In order to obtain clinical evidence of the allergic factor in GER, especially in older children (over 3 years of age), constitutional features of allergy according to Marks were considered [35].

Once the diagnosis had been made, proper therapy was instituted and clinical observation was recommended at 6-week intervals (prospective studies).

Each patient was subjected to physical examination at every check-up. General condition and competence of systems and organs, especially those at risk of GER effects, were assessed.

Results

As shown in *Tab. 3*, of 735 preliminary patients, 264 (35.9%) children of both genders with suspected gastroesophageal reflux disease and family history of gastrointestinal disorders were qualified for the study.

The 24 h pH-metric testing of the oesophagus, endoscopic examination of the upper gastrointestinal tract and histopathological investigation of oesophageal mucosa specimens confirmed acid gastroesophageal reflux in 170 (23.1%) patients. GERD was diagnosed in 138 of them (18.8%), including 54 (7.3%) with reflux oesophagitis. In the remaining 565 (76.9%) children, including 94 (12.8%) with family history of gastroin-

Table 3. **Examination results in 735 children with suspected gatsroesophageal reflux disease (GERD), hospitalized in the Department of Children Diseases in Białystok, in the years 1992-1995**

Diagnostic investigation	Study children		Acid gastroesophageal reflux									
			present								absent	
			pathological						physiological			
			total		oesophageal reflux disease		reflux inflammation of oesophageal mucosa					
	N	%	N	%	N	%	N	%	N	%	N	%
24-hour oesophageal pH-metry	735	100.0	138*	18.8*	84	11.4	-	-	32*	4.3*	565 94* 471	76.9 12.8* 64.1
Endoscopic examination of oesophagus, stomach and duodenum	703	95.6			-	-	54	7.3			649**	88.3**

* A total of 264 (35.9%) children with suspected oesophageal reflux disease and positive family history of gastrointestinal disorders;
** In these patients, inflammation of oesophageal mucosa was not macroscopically or histopathologically confirmed

testinal disorders, neither pathological GER nor inflammatory changes in the oesophageal mucosa were found.

Positive oral food challenge test confirmed secondary pathological GER in 62 children (8.4%), mean age x=21.53 months ±17.79.

In the remaining 76 (10.3%) children, mean age x=25.20 months ±27.28, primary GER was diagnosed.

Discussion

Recent years have brought an increase in the number of clinical observations indicating that gastroesophageal reflux (GER) may have an allergic background.

In children, especially in the youngest (infancy), cow milk allergy (cma) is the most common cause of GER [6,7,9,10,18--20].

In older children, GER can be a clinical manifestation of body hypersensivity to diary meals, other food products and inhaled air allergens [36-38].

The causal relationship between these two pathological conditions, i.e. GER and cow milk allergy is indicated by age (most frequently infancy) and similarity of clinical symptoms typical of both pathologies [1,6].

Forget et al. have suggested that these two disorders are most frequent in the first months of life and tend to disappear after the first year of life. According to these authors, their incidence is similar, ranging from 1% to 10% [39].

Hill et al., studying the diversity of symptoms in the course of hypersensitivity to cow milk proteins, revealed that 6% of the youngest patients had gastroesophageal reflux symptoms [40].

However, other authors have reported that the percentage of secondary GER in the youngest children with cow milk allergy is substantially higher and reaches 16-40% [6,7,18].

In a group of vomiting infants, Staiano et al. found pathological acid GER to coexist with cow milk allergy in 16% of the children and cow milk allergy alone in the same percentage of patients [18].

Cavataio et al. in prospective studies observed a co-occurrence of cow milk allergy in 41.7% infants with acid GER [6,7]. The authors, comparing the symptoms of secondary and primary GER found no differences in age, gender or clinical picture between these groups of patients.

Kaczmarski et al. were the first in Poland to make similar clinical observations indicating the causal role of food allergy in triggering acid GER [3,8,9,16].

In the current study, the share of allergy to cow milk proteins and/or other food products in secondary GER accounted for 36.5%, while in GERD for 44.9% of children at various age.

We instituted our own algorythm of diagnostic and therapeutic management in order to properly differentiate primary (idiopathic) from secondary GER. As food allergy was considered to be a potential cause of GER, temporary elimination of harmful food products was introduced for the period of 4-6 weeks.

Then, the allergic patients who responded well to elimination diet (regression of secondary GER symptoms), were subjected to challenge test with cow milk and/or other food product to supply evidence for the cause-and-effect relationship. Depending on the patient's age and type of clinical symptoms, the challenge test was either open or blind, placebo-controlled. [16,32].

The recurrence of symptoms during challenge test confirmed the diagnosis of food allergy and its share in secondary GER.

In addition to elimination diet and challenge test, complete differential diagnosis of GER is essential and includes allergological and immunological investigations, radiological examinations of the chest, upper gastrointestinal tract, lateral sinuses of the nose, as well as biochemical, metabolic, bacteriological studies and others [2,4,9,10].

In the children with a causal role of food hypersensitivity in triggering GER, elimination diet was found to have high nutritional and therapeutic values both in the treatment of allergy and GER, and in some of the patients no antireflux drugs had to be administered.

However, when complete improvement is not achieved, antiallergic and eventually antireflux drugs should be administered [6-8,10].

Those children who did not present with a recurrence of symptoms during food challenge had primary type of GER and underwent a classic variant of antireflux treatment (prokinetic drugs and/or regulating gastric acid secretion) [2,8,10,15,17,41,42].

Conclusion

The current study revealed the existence of the cause-and--effect relationship between allergy to cow milk protein/other food products and GER in the study children at various age.

Acknowledgement

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Gastroesophageal reflux in children and adolescents. Clinical aspects with special respect to food hypersensitivity

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Abstract

Purpose: Gastroesophageal reflux (acid GER), primary and secondary, has a wide spectrum of clinical symptoms and occurs at developmental age.

The study objective was to elaborate the clinical profile of symptoms and to determine whether there are any differences in clinical manifestations between the two acid GER conditions, i.e. primary and secondary to cow milk allergy and/or other food allergy (CMA/FA).

Material and methods: The study involved 264 children of both genders and at various age, with diverse reflux symptoms from one or many organs and with a positive family history of alimentary tract diseases. Based on preliminary diagnostic tests, the children were divided into groups. In 138 children, pathological acid GER, primary and secondary to CMA/FA, was recognized.

Results: The profile of clinical symptoms observed in 264 children with suspected gastroesophageal reflux disease (GERD) was elaborated according to their frequency. Among differentiating symptoms the most common were: vomiting (12.1%), bronchitis (10.9%) and pneumonia (9.6%). In the group of 138 GER children, 32 (23.2%) had monosystemic symptoms, in the other 106 patients (76.8%) many systems were affected. The most frequent reflux symptoms were: in primary GER (group 2) – alimentary tract disorders (28.6%), pneumonia and bronchitis (20.7%) and neurological symptoms with torticollis (7.4%); in secondary GER (group 3) – alimentary tract disorders: vomiting and anxiety/crying (25.2%); pneumonia and bronchitis (19.4%). In 23 children (37%) with secondary GER, typical allergic symptoms were found to coexist. The 138 GER patients underwent allergologic and immunologic tests to confirm the allergic background of symptoms.

Conclusion: Clinical symptoms caused by the presence of secondary acid GER are non-specific, being identical or similar to those observed in primary acid GER. Allergologic and immunologic tests are useful to confirm or exclude the relationship between GER and CMA/FA in the study children.

Key words: acid gastroesophageal reflux: primary, secondary; food allergy; clinical manifestation; allergologic and immunologic tests; children.

Introduction

According to the generally accepted definition, gastroesophageal reflux (acid GER) is the involuntary, temporary or permanent passage of gastric contents into the esophagus as the result of defective functional competence of the respective elements of the antireflux barrier, mainly of the lower esophageal sphincter (LES) with its abnormal resting tension and disturbed motor activity of the upper alimentary tract [1-7].

Pathogenetically, reflux can be divided into primary GER (physiologic or pathologic), secondary GER and gastroesophageal reflux disease (GERD) [8-12].

Gastroesophageal reflux disease (GERD) is a clinical condition manifested by differentiating symptoms due to diagnostically confirmed pathological GER (primary or secondary).

Reflux esophagitis (RE) is the most common local complication of GERD [2,13-16].

Numerous in-depth clinical observations conducted by various authors on the population of developmental age and adults have confirmed that GER is responsible for complex and abundant clinical symptomatology, with different organs and systems involved (alimentary tract, respiratory system, circulatory system, central nervous system, all-systemic ailments) in isolated or associated form.

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Table 1. **A list of reflux symptoms [17]**

Reflux symptoms	
Typical	**Atypical**
– regurgitation	– hoarseness
– vomiting	– laryngitis (stridor)
– hematemesis and/or melaena	– obturative bronchitis (wheezing)
– body weight and/or height deficiency	– spastic bronchitis
– abdominal pain	– pneumonia
– retrosternal pain	– persistant cough
– food refusal	– asthmatic syndrome
– heartburn	– anemia
– hiccups/belching	– changes in musle tension of the nape and/or trunk (arching, myotonia)
– irritability, anxiety/paroxysmal crying	– Sandifer's syndrome
– dysphagia	– ALTE syndrome (apnea, cyanosis, pallor, consciousness disturbances, bradycardia)
– odynophagia	

Table 2. **Overall presentation of types and frequency of clinical symptoms of esophageal reflux disease with respect to age (according to Salvatore et al.) [23]**

Reflux symptoms	Infants	Children	Adults
Vomiting	++	++	+
Regurgitation	++++	+	+
Heartburn	?	++	+++
Abdominal pain	?	+	++
Retrosternal pain	?	+	++
Dysphagia	?	+	++
Paroxysmal irritability/crying	+++	+	-
Anemia	+	+	+
Body weigh deficiency	++	+	-
Abnormal posturing (Sandifer's s.)	++	+	-
Hiccups	++	+	+
Dental erosion (caries)	?	+	+
Hoarseness/aphonia	?	+	+
Chronic cough/bronchiopulmonary symptoms	+	++	+
Wheezing/laryngitis/ear problems	+	++	+
Laryngomalacia/stridor	+	++	-
Asthmatic syndrome/sinusitis	-	++	+
Laryngostenosis	-	+	+
ALTE (SIDS/apnea)	+	-	-
Bradycardia	+	?	?
Sleep disturbances	+	+	+
Impaired quality of life	++	++	++
Esophagitis	+	+	++
Esophageal stenosis or shortening	-	(+)	+
Barrett's esophagus/adenocarcinoma	-	(+)	+

++++ – predominant; +++ – very common; ++ – common; + – possible; (+) – rare; - – absent; ? – unknown

Most symptoms fall into two categories: typical and atypical ones [3,17-22]. The spectrum of acid GER symptoms has been presented in *Tab. 1* [17].

Salvatore et al. carried out a more up-to-date clinical analysis of reflux symptoms (2004) according to patients' age (*Tab. 2*), showing a considerable similarity in their type and differentiation in frequency in comparison to earlier overall analyses of this type [23].

In approximately 30% of children, enhanced passage of gastric contents into the esophagus becomes a pathology (primary or secondary) and usually causes troublesome symptoms, mainly from the esophagus (typical) [1,11,13,21,22].

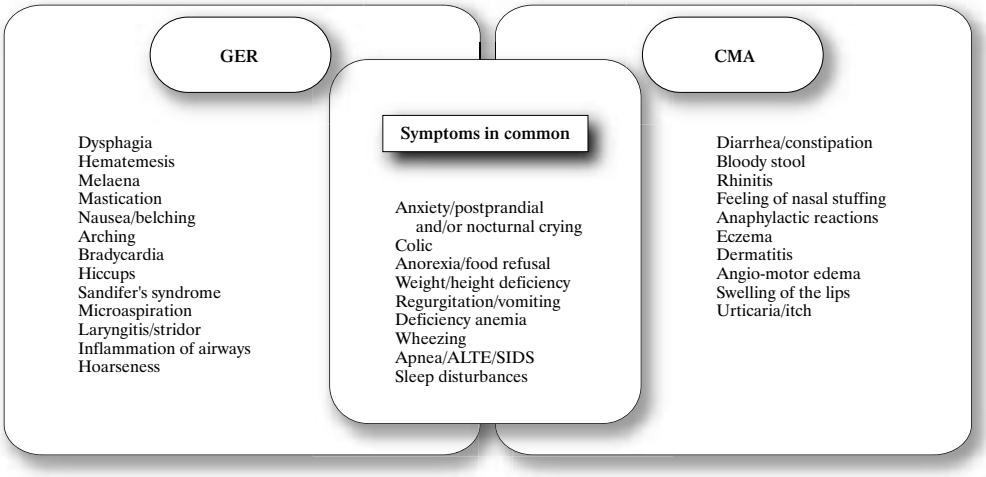
GER can also be responsible for a number of differentiated atypical symptoms, with respiratory, circulatory, neurological or all-systemic manifestations [2,7,13].

Duration of reflux symptoms varies, as they are usually chronic or recurrent.

Secondary GER is a pathologic phenomenon that usually occurs in the course of already existing disorders, such as infectious, allergic, neurologic, systemic, genetic, metabolic and others [2,11,13].

Clinical symptoms of GER may have a causal association or may coexist with food hypersensitivity, especially with allergy to cow milk proteins (mainly in infancy) and/or other food prod-

Figure 1. **Profile of clinical symptoms in the course of GER and CMA [13]**



ucts commonly consumed by older children (secondary reflux) [10,11,13].

Potential ethiopathogenic relationships between GER and CMA, the most common early childhood ailments, have been investigated for a few years now [8-10,24].

Not only the age of patients, but also the whole range of clinical symptoms these two entities have in common (e.g. regurgitation/vomiting, colic, anxiety, insufficient weight increase and others) (*Fig. 1*), seem to justify an attempt to prove the existence of mutual relations [13].

In older children, GER which is clinical manifestation of food hypersensitivity presents with such differentiated symptoms as vomiting/regurgitation, ruminatio, belching/hiccups, abdominal pain, wheezing, paroxysmal cough, bronchitis, hoarseness and others [2,6,7,23].

However, literature evidence for the association between GER and food hypersensitivity in older children is scarce [9--12,24-26].

The study objective was to: elaborate a clinical profile of symptoms (similarities and differences) in children with secondary acid GER, having a causal association with food hypersensitivity, and with primary acid GER.

Material and methods

A total of 735 children with suspected GERD, hospitalized in III Department of Pediatrics, Medical University of Białystok in the years 1992-1995, underwent diagnostic examinations (24--hour intraesophageal pH-metry and endoscopic examination of the upper alimentary tract). Among them, the major group consisted of 264 (35.9%) children of both genders, with differented mono- and polysystemic symptoms and with a positive family history of alimentary disorders.

Allergologic and immunologic tests were performed in children with diagnosed pathologic acid GER to confirm its allergic cause (secondary GER) and to differentiate it from primary GER (idiopathic).

a) Skin "prick tests" – done with:

– a set of native food allergens (fresh);

– a set of inhalatory commercial allergens (Smith Kline Beecham – USA).

These tests were performed once in 71 children with pathologic GER and in 32 patients with only CMA/FA at various age in order to confirm or exclude early IgE-dependent hypersensitivity to food and/or inhalatory allergens (atopic factor).

b) Total serum (tIgE)

Total serum IgE was determined using Fluoro-FAST method (3M Diagnostic Systems, USA) in 170 children and this determination was significantly helpful in differentiation of pathogenetic IgE-dependent and independent mechanisms involved in food allergy. The tIgE level in serum was considered elevated when its value was >50 IU/ml.

As a single measurement of total IgE has a limited specificity in atopy recognition, other specific immunoglobulins of this class have been determined for chosen food allergens.

c) Qualitative and quantitative assessment of specific IgE against food allergens (a-s IgE) and inhalatory allergens (i-s IgE), with Fluro-FAST method (3M Diagnostic Systems, USA).

Determination of allergen-specific immunoglobulins in the study children not only confirmed IgE-dependent pathomechanism of food allergy, but they also allowed recognition of sensitizing food allergens. It was also helpful whenever, for various reasons, prick tests could not be performed or their results were doubtful.

We performed qualitative and quantitative assessment of a-s IgE/and i-s IgE in 103 patients with suspected allergy, including those with positive prick tests, with food and/or inhalatory allergens and elevated serum IgE.

Specific IgE were those in class 2-5 and included:

– a-s IgE against cow milk proteins, hen egg white, soya protein, fish protein, oranges;

– i-s IgE against grass pollen, tree pollen, bush and weed pollen, house dust mites and cat fur, determined in serum.

d) Eosinophilia

Eeosinophilia was assessed once in peripheral blood smear

Table 3. **Study results in 735 children with suspected gastroesophageal reflux disease (GERD), hospitalized in III Department of Pediatrics in the years 1992-1995**

Diagnostic investigation	Study children		Gastroesophageal reflux									
			Present								Absent	
			Pathologic						Physiologic			
			Total		Esophageal reflux disease		Reflux mucosal esophagitis					
	N	%	N	%	N	%	N	%	N	%	N	%
24-hour intraesophageal pH-metry	735	100.0	138*	18.8*	84	11.4	-	-	32*	43*	565 *94**	76.9 *471* *12.8** *64.1*
Endoscopy of esophagus, stomach and duodenum	703	95.6			-	-	54	7.3			*649***	*88.3***

*A total of 264 (35.8 %) children with GERD and positive family history of alimentary disorders.
** Mucosal esophagitis was not confirmed macroscopically or histopathologically in these patients.

Table 4. **Classification of 264 children with suspected gastroesophageal reflux disease into study groups (at the time of diagnosis)**

Study groups	Gender	Study children with reflux symptoms							
		Number		Age range in months					
				>1.5 m – 4 m		>4 m – 16 m		>16 m – 102 m	
		N	%	N	%	N	%	N	%
Group 1 N=32 Physiologic GER	Boys	17	6.4	17	6.4	-	-	-	-
	Girls	15	5.7	15	5.7	-	-	-	-
Group 2 N=76 Primary GER	Boys	39	14.8	-	-	23	8.7	16	6.0
	Girls	37	14.0	-	-	21	7.9	16	6.0
Group 3 N=62 GER + CMA/FA	Boys	33	12.5	-	-	16	6.1	17	6.4
	Girls	29	11.0	-	-	14	5.3	15	5.7
Group 4- reference N=32 CMA/FA	Boys	19	7.2	-	-	7	2.6	12	4.5
	Girls	13	4.9	-	-	5	1.9	8	3.0
Group 5 N=62 GER (-) + CMA/FA (-)	Boys	32	12.1	-	-	8	3.0	24	9.1
	Girls	30	11.4	-	-	10	3.8	20	7.6
Total		264	100.0	32	12.1	104	39.4	128	48.5

in 138 children with pathologic GER and in 32 patients with CMA/FA alone.

It was a useful laboratory indicator confirming the allergic cause of symptoms from the alimentary tract and other organs.

The percentage value of eosinophilia was treated as abnormal when >5%.

Classification of patients into study groups

Based on preliminary diagnostic tests carried out in 264 children, as well as using dietary analysis and the findings obtained from elimination diet test and oral food challenge in 138 of them, the children were classified into study groups (*Tab. 3, 4*; *Fig. 2*).

Of 170 (64.4%) patients with diagnosed acid GER (89 boys, 81 girls), 32 (12.1%) of the youngest infants with physiologic GER (group 1; mean age x=2.2 months ±0.48) were excluded from further analysis and clinical observation.

The remaining 138 (52.3%) children had primary and secondary pathologic GER, and were classified into groups 2 and 3.

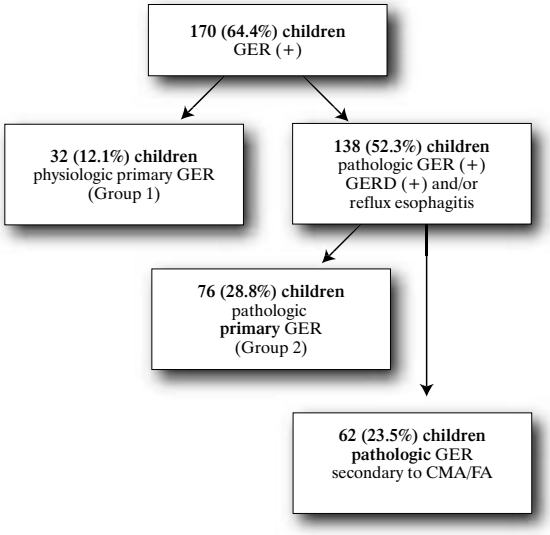
Group 2 included 76 (28.8%) patients (39 boys – 14.8%; 37 girls – 14.0%), aged 4-102 months (mean age x=25.20 m ±27.28), with pathologic primary GER.

Group 3 contained 62 (23.5%) patients (33 boys – 12.5%; 29 girls – 11.0%), aged 4-74 months (mean age x=21.53 m ±17.79), with secondary pathological GER. Ninety-four (35.6%) of 264 patients with symptoms suggesting GERD, positive family history of alimentary disorders and with non-confirmed pathologic GER were classified into:

– group 4 – 32 (12.1%) patients (19 boys – 7.2%; 13 girls – 4.9%) aged 7 months – 69 months (mean age x=23.70 m ±12.63) with pathologic symptoms against a background of diagnosed allergy to cow milk proteins and/or other food products (the so called reference group);

– group 5 – 62 (23.5%) patients (mean age x=31.3 months

Figure 2. **Further classification of 170 children with diagnosed acid GER into study groups. Prospective clinical observation with the use of differentiating diagnostic-therapeutic algorythm**



±27.98), with non-confirmed reflux or allergic cause of the existing symptoms. These children were excluded from further analysis and clinical observation.

Patients classified into the above groups did not differ significantly (p=ns) with respect to age and gender.

Results

Clinical profile of the study children

Twenty-four clinical reflux symptoms observed in 264 children with GERD and a positive family history of alimentary disorders according to their frequency have been presented in *Tab. 5*. Among them the most common were: vomiting (12.1%), bronchitis (10.9%) and pneumonia (9.6%), the least common being anemia (1.3%), apnea (1.0%) halitosis (1.0%), nasal obstruction (coryza) (0.6%), and dysphagia (0.4%).

The frequency of pathologic acid GER-dependent symptoms, isolated or associated from the alimentary tract, respiratory system and nervous system in 138 children, has been presented in *Tab. 6*.

Table 5. **Type and frequency of clinical symptoms in the study children (suspected esophageal reflux disease, positive family history of alimentary ailments)**

Clinical symptoms			
No	Type	Incidence	
		N=478	%
1.	Vomiting	58	12.1
2.	Bronchitis	52	10.9
3.	Pneumonia	46	9.6
4.	Abdominal pain	39	8.2
5.	Regurgitation	32	6.7
6.	Food refusal	32	6.7
7.	Ruminatio	31	6.5
8.	Anxiety/crying	23	4.8
9.	Neurological symptoms*	21	4.4
10.	Obturative bronchitis	21	4.4
11.	Weight deficiency	19	4.0
12.	Chronic cough	15	3.1
13.	Belching / hiccups	12	2.5
14.	Coexistence of vesicoureteral reflux	12	2.5
15.	Heartburn	11	2.3
16.	Pharyngitis / laryngitis	9	1.9
17.	Spastic bronchitis	7	1.5
18.	Wheezing / dyspnea and/or paroxysmal cough	8	1.7
19.	Torticollis**	8	1.7
20.	Anemia	6	1.3
21.	Apnea	5	1.0
22.	Halitosis (foetor ex ore)	5	1.0
23.	Nasal obstruction (coryza)	3	0.6
24.	Dysphagia	2	0.4

* – arching, myotonia, cyanosis, pallor, disturbances of consciousness; ** – coexistence with neurological symptoms

Isolated symptoms (typical) from the alimentary tract were found in 24 (17.4%) children, from the respiratory system (atypical) in 8 (5.8%). In total, 32 (23.2%) of 138 young patients had monosystemic GER symptoms. The remaining 106 (76.8%) showed polysystemic symptoms (associated) from the alimentary, the respiratory and the central nervous system (92; 66.7%), the alimentary and the nervous system (9; 6.5%), and the respiratory and the nervous system (5; 3.6%).

Table 6. **Frequency of isolated and associated ailments from various organs/systems in 138 children with pathologic acid GER: primary (group 2) and secondary to cow milk proteins and/or other foods (group 3)**

Monosystemic disorders			Polysystemic disorders		
Type of disorder	N	%	Type of disorder	N	%
Alimentary system*	24	17.4	alimentary system + respiratory system***	81	58.7
			alimentary system + respiratory system + nervous system ****	11	8.0
Respiratory system **	8	5.8	alimentary system + nervous system *****	9	6.5
			respiratory system + nervous system	5	3.6
Total	32	23.2	138 – 100.0 %	106	76.8

*[r] with vesicoureteral reflux – 1 (0.7 %) child; ***[r] with vesicoureteral reflux - 8 (5.8 %) children; ***[a] with asthma – 9 (6.5 %) children; *[n] with anemia – 3 (2.2 %) children; **[n] with anemia – 3 (2.2 %) children; ****[s] with Sandifer's syndrome – 4 (2.9 %) children; *****[s] with Sandifer's syndrome – 4 (2.9 %) children; *[b] with Barrett's esophagus – 1 (1,3 %) children

Table 7. **Comparative analysis of type and frequency of clinical symptoms in study groups of children with pathologic acid GER: primary (group 2) and secondary to CMA/FA (group 3)**

Primary gastroesophageal reflux (2)			Secondary gastroesophageal reflux (3)		
Clinical symptoms					
Type	Frequency		Type	Frequency	
	N=188	%		N=139	%
Pneumonia	20	10.6	Vomiting	20	14.4
Bronchitis	19	10.1	Bronchitis	16	11.5
Regurgitation	15	8.0	Anxiety/crying	15	10.8
Vomiting	14	7.4	Neurological symptoms	13	9.3
Masticatio (Ruminatio)	14	7.4	Pneumonia	11	7.9
Food refusal	11	5.8	Food refusal	10	7.2
Obturative bronchitis	10	5.3	Abdominal pain	9	6.5
Weight deficiency*	10	5.3	Wheezing/dyspnea and/or paroxysmal cough	8	5.8
Coexistence of vesicoureteral reflux	9	4.8	Weight deficiency	6	4.3
Abdominal pain	8	4.2	Regurgitation	5	3.6
Belching/hiccups	8	4.2	Masticatio (Ruminatio)	5	3.6
Heartburn	8	4.2	Chronic cough	5	3.6
Anxiety/crying	8	4.2	Obturative bronchitis	4	2.9
Chronic cough	8	4.2	Heartburn	3	2.2
Neurological symptoms	8	4.2	Anemia	3	2.2
Torticollis	6	3.2	Halitosis (foetor ex ore)	2	1.4
Halitosis (foetor ex ore)	3	1.6	Torticollis	2	1.4
Spastic bronchitis	3	1.6	Spastic bronchitis	1	0.7
Anemia	3	1.6	Belching/hiccups	1	0.7
Dysphagia	2	1.1	-	-	-
Apnea	1	0.5	-	-	-
Coexisting allergic symptoms N=23			Generalized dermatitis	9	39.1
			Urticaria	4	17.4
			Rhinitis	4	17.4
			Itch	3	13.0
			Chronic diarrhea	3	13.0

*Barrett's esophagus – in 1 child

Tab. 7 presents a comparative analysis of the type and frequency of clinical symptoms found at the time of diagnosis in the study groups of 138 children with pathological acid GER: primary (group 2) and secondary to CMA/FA (group 3).

The most common reflux symptoms included:

– in primary GER (group 2) – alimentary manifestations in the following order: vomiting, ruminatio, food refusal (28.6% in total); pneumonia and bronchitis (20.7% altogether), and neurologic symptoms with torticollis (7.4%);

– in secondary GER (group 3) – alimentary manifestations: vomiting and anxiety/crying (25.2% in total); bronchitis and pneumonia (19.4% in total).

Less common were:

– in group 2 – obturative bronchitis and body weight deficiency (with the same frequency 5.3%), as well as other symptoms, including abdominal pain, belching/hiccups, heartburn and anxiety/crying (16.8% altogether), and chronic cough (4.2%);

– in group 3 – typical symptoms, such as anorexia, abdominal pain and weight deficiency, regurgitation and rumination (with the same frequency; 25.2% altogether); wheezing with dyspnea and/or paroxysmal cough, chronic cough and obturative bronchitis (12.3% althogether).

The least frequent symptoms included:

– in group 2 – halitosis (foetor ex ore), spastic bronchitis and anemia (with the same frequency 1.6%); moreover, dysphagia (1.1%), exceptionally apnea attacks (0.5%);

– in group 3 – with the same frequency: heartburn and anemia (2.2% each), halitosis (foetor ex ore) and torticollis (1.4% each), very seldom spastic bronchitis and belching/hiccups (0.7%).

Nine children (11.8%) with primary GER (group 2) were additionally diagnosed with vesicoureteral reflux.

Interesting is that in 23 (37.0%) children with secondary GER (group 3) non-reflux allergic symptoms were found to coexist.

All the ailments were almost similar and their frequency did not differ much between both groups.

Results of allergic and immunologic tests in 138 study children with pathologic acid GER

Results of allergic and immunologic tests performed in 138 study children with pathologic acid GER at the time of diagnosis

Table 8. **Results of allergologic and immunologic tests in 138 children with pathologic acid GER (at the time of establishing diagnosis)**

Type of examination		Study children			
		Primary GER (group 2, n=76)		GER secondary to CMA/FA (group 3, n=62)	
		N	%	N	%
Positive family history of allergy		9	11.8	43	69.4
Atopy in family		3	3.9	25	40.3
Positive skin-prick tests with allergens	native food allergens	0	0.0	28	45.2
	commercial inhalatory allergens	4	5.3	8	12.9
	food and inhalatory allergens	0	0.0	20	32.3
tIgE levels	>50-100 IU/ml	3	3.9	18	29.0
	>00 IU/ml	0	0.0	16	25.8
sIgE (class 2-5) against:	food allergens	0	0.0	17	27.4
	inhalatory allergens	0	0.0	4	6.5
	food and inhalatory allergens	0	0.0	8	12.9
Eosinophilia of peripheral blood	>5 – 8%	5	6.5	24	38.8
	>8 – 15%	0	0.0	18	29.0
Constitutional features (clinical hallmarks) of allergy – (min.2)		5	6.6	19	30.6

were analyzed in order to confirm the allergic cause of clinical symptoms (*Tab. 8*).

In group 3 consisting of 62 children with GER secondary to CMA/FA, most patients (43; 69.4%) had a positive allergic family history (allergy found among the closest relatives). This feature was the least common in group 2, i.e. in only 9 patients out of 76 with pathologic primary GER (11.8%).

Diverse allergic – non-reflux symptoms coexisted in 23 (37.1%) of 62 children in group 3, but were absent in group 2 patients.

Constitutional features of allergy were observed most often in group 3 – 19 patients (30.6%) as compared to 5 (6.6%) from group 2. In group 2, three children had elevated level of total IgE (tIgE) and in two eosinophilia was increased.

Positive results of skin-prick tests: with food allergens were obtained in 28 children (45.2%) in group 3.

Positive results of tests with food and inhalatory allergens referred to 20 patients (32.3%) in group 3.

Positive results of tests with only inhalatory allergens were found in 8 children (12.9%) from group 3 and in 4 (5.3%) from group 2, all with a positive family history.

Total serum IgE level in peripheral blood was elevated (>50-100 IU/ml) in 18 patients (29.0%) from group 3. Only 3 children (3.9%) from group 2, with a positive family history of allergy, showed increased tIgE level (values in the range above).

The values of tIgE >100 IU/ml were found in 16 patients (25.8%) from group 3.

The presence of specific IgE (sIgE) in the serum was detected in 29 children (46.8%) from group 3. They were positive (class 2-5) against food allergens – in 17 (27.4%); against food and inhalatory allergens – in 8 (12.9%) and against inhalatory allergens alone – in 4 (6.5%) children.

Most frequently identified food allergens included cow milk proteins, hen egg white, soya protein, fish protein and oranges. Grass pollen, tree pollen, bush and weed pollen, house dust mites and cat fur were the most common inhalatory allergens.

Elevated serum relative eosinophilia in peripheral blood within the range of values >5-8% was observed in 24 patients (38.8%) from group 3 and in 5 (6.5%) from group 2.

Relative eosinophilia in the range of values >8-15% was found in 18 (29.0%) children from group 3.

Atopy (positive family history of allergy and elevated serum tIgE) was diagnosed most frequently in 25 children with secondary GER (40.3%) from group 3, being the least common in group 2 children with primary GER (3; 3.9%).

Discussion

Allergy to cow milk protein or to other food products in children, irrespective of their age, is one of the more frequently recognized causes of secondary GER, although so far this relationship has been rarely described in clinical reports [10,24,25,27].

The first and fundamental step in making a decision about antireflux treatment is differentiation between primary (idiopathic) GER and secondary (to food hypersensitivity) GER. To do this we implemented our own algorythm of diagnostic-therapeutic procedure [9,16,26,28-31].

As revealed by our observations and clinical investigations performed on a relatively large group of infants in the first year of age, cow milk allergy (CMA) is the cause of secondary GER in 43% of cases [12]. These investigations also confirm that the application of cow milk elimination diet considerably improves clinical condition of children with CMA and GER; thus, the coexistence of these two pathologic entities is not coincidental [12].

Our findings have been confirmed by the results published by the Italian researchers who found secondary GER in infants with CMA in 16-40% of cases [8,10,32].

In a group of vomiting infants, Staiano et al. revealed pathologic GER coexisting with CMA in 16% and CMA alone in the same percentage of cases [8].

Cavataio et al., in prospective studies, found coexistence of CMA and GER in 41.7% [10,32].

At the same time, these authors did not reveal any significant differences in age, gender or clinical picture between children with primary and secondary GER.

In the current study, conducted on a group of children at various age, the share of cow milk and/or other food allergy in initiation of secondary GER was estimated at 36.5%, accounting for 44.9% in gastroesophageal reflux disease (GERD). Clinical manifestation (type of reflux symptoms, mono- or polysystemic, frequency in isolated or associated form) in children with GER secondary to CMA/FA showed no significant differences as compared to that observed in primary GER.

However, it should be emphasized that 37% of children with both CMA/FA and GER developed typical allergic symptoms, such as generalized dermatitis, urticaria, rhinitis, itching of the skin, chronic diarrhea.

Obviously, the children with CMA/FA showing remission of secondary GER following elimination diet should undergo oral challenge test with cow milk and/or another harmful food product (open or blind according to the patient's age and types of clinical symptoms) to confirm the cause-and--effect relationship between these two pathologic conditions [26,30].

However, a positive family history of allergic diseases, clinical evidence of allergy, i.e. its constitutional features, allergologic and immunologic tests and their proper interpretation appear to be very useful and helpful in establishing definite diagnosis and confirming the allergic cause of detected GER in children [2,9,12,13,25,26,33].

Conclusions

1. Coexistence of cow milk allergy and food allergy (CMA/FA) with esophageal reflux disease was found in 44.9% of children at various age.

2. Clinical symptoms caused by acid GER associated with CMA/FA (secondary), irrespective of age, are nonspecific and similar to or identical with those observed in primary acid GER.

3. Commonly available and accepted allergologic and immunologic tests should be used to confirm or exclude the relationship between GER and CMA/FA in children; positive results indicate IgE-dependent pathogenetic mechanism underlying clinical symptoms. However, it is the positive food challenge that is a decisive test.

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Optimal maintenance therapy in patients with non-erosive reflux disease reporting mild reflux symptoms – a pilot study

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Abstract

Purpose: This pilot study aimed at finding trend for further investigation of the optimal maintenance therapy with lansoprazole in patients with non-erosive reflux disease (NERD) suffering from mild symptoms.

Material and methods: Sixty consecutive patients with diagnosed NERD reporting mild symptoms were included in the study. After successfully finishing a four-week treatment with lansoprazole (30 mg daily), the patients were randomized into three groups administered: 1 – lansoprazole 30 mg "on--demand", 2 – lansoprazole 15 mg daily, 3 – lansoprazole 30 mg in four-week courses during a relapse. The intensity of symptoms was assessed with the Visual Analogue Scale (VAS) at the baseline, as well after 4 weeks, 3, 6 and 12 months of therapy. The general satisfaction of treatment was evaluated with the Verbal Rating Scale (VRS) at the same time.

Results: At the baseline, the mean intensity of symptoms assessed by VAS was 2.8±1.0 points and fell to 0.4±0.5 points after a 4-week therapy. In Group 1, after 3, 6 and 12 months, it was 0.85±0.6, 1.0±0.8 and 1.0±0.8, in Group 2: 0.65±0.7, 0.65±0.7, 0.5±0.3, and in Group 3: 1.1±0.6, 1.55±0.7, 1.65±0.8 points, respectively. No significant differences were observed between Groups 1 and 2. Intermittent therapy (Group 3) showed a significantly lower efficacy in comparison to other groups ($p<0.05$). "On-demand" therapy was 30% cheaper whereas intermittent therapy was 55% cheaper than the most expensive daily treatment. However, general satisfaction of treatment assessed by VRS was non-significantly different between any of the groups.

Conclusions: In patients with NERD and mild symptoms, both on-demand and daily treatment models of maintenance therapy showed a similar high efficacy, whereas intermittent therapy was significantly less effective. However, general satisfaction of each treatment options was high and non-significantly different between the groups. Due to a pilot character of this study further investigation based on a larger number of patients is necessary to confirm the clinical value of cheaper models of maintenance therapy which could be then recommended as more cost-effective.

Key words: NERD, "on-demand" therapy, intermittent therapy.

Introduction

Complaints typical for gastroesophageal reflux disease (GERD) are experienced daily by 10% of adult population, but as many as 20-40% of adults suffer from such symptoms at least once a month. In the majority of patients (50-70%), no inflammatory lesions are detected in the esophagus and such individuals are diagnosed as suffering from the so-called non-erosive reflux disease (NERD). In view of its persistent and recurrent character, GERD contributes to poorer quality of life in numerous patients. In addition, some individuals with NERD manifest an increased sensitivity to acid, what may lead to a weaker reaction to inhibition of hydrochloric acid secretion as compared to patients with confirmed esophagitis. The objective of an optimum, long-term therapy is the improvement of the quality of life, prevention of complications, as well as prevention of recurrent disease [1-3]. It is estimated that as early as within six months of discontinuing regular administration of proton pump inhibitors (PPI), approximately one half of the patients (40-60%) again develop reflux symptoms. The risk of a recurrent disease is at its maximum in the first year after the diagnosis. The initial phase of the therapy includes the administration

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of PPI once or twice a day continued for approximately 4 to 8 weeks [3,4]. In maintenance therapy, several management models are currently proposed, such as the "step-down" therapy, "on-demand" therapy, intermittent therapy that is employed when the complaints recur, as well as long-term therapy with low PPI doses [4-8]. In view of the high heterogeneity of patients with GERD, their therapeutic requirements may also differ.

To-date, there is no clarity as to which of the above therapeutic protocols is optimal with respect to its efficacy and cost in view of pre-therapeutic complaint intensity in patients with NERD [9].

The objective of this pilot study is determining trend for further investigation of the most optimal method of long-term treatment of reflux disease in patients with NERD who report mild complaints based on three models of pharmacological management using PPI: "on-demand" therapy, maintenance therapy and intermittent therapy. Additionally, the authors evaluate the costs incurred by the employment of particular therapeutic methods.

Material and methods

The study included 65 consecutive patients (36 females and 29 males aged 18 to 71 years, 48.6 in average) with non-erosive reflux disease diagnosed based on characteristic clinical presentation (heartburn, belching, regurgitation) and endoscopic examinations seen at the Outpatient Clinic, Department of Gastroenterology and Hepatology, University Hospital of Cracow, Poland. The inclusion criterion consisted in mild reflux symptoms (baseline intensity of symptoms 4 or less points on VAS) that would not affect daily activities of the patients and persisted for at least three months prior to the visit.

The following patients were excluded from the study: individuals with severe systemic diseases, esophagitis, esophageal ulceration, esophagostenosis, peptic ulcers, past surgery involving the upper gastrointestinal tract or reporting complaints, which in the opinion of the investigators might suggest the irritable bowel syndrome or dyspepsia, medication with any drugs that influence either the lower esophageal sphincter motility or gastric secretion and emptying.

A detailed medical history was taken in all the patients and upper GI tract endoscopy was performed.

The investigation consisted of two stages: stage 1 included a four-week PPI treatment, while stage 2 was an 11-month follow-up. Having completed the preliminary, four-week therapy with PPI (lansoprazole) administered at the dose of 30 mg once a day, the patients in whom the treatment had been successful (success being defined as no complaints whatsoever or not more than one day with mild complaints within 7 days immediately prior to the assessment) were randomized (with sealed envelopes method) to three groups equal in number (n=20, each):

1. Group 1, administered 30 mg lansoprazole as needed ("on-demand" therapy);
2. Group 2, receiving a daily maintenance dose of 15 mg of lansoprazole;
3. Group 3, on a four-week course of lansoprazole at the dose of 30 mg, in case of recurrent symptoms (intermittent therapy).

The patients were asked to report to their physician should their symptoms recur, as well as after 3, 6 and 12 months of therapy. The intensity of symptoms was rated each time using the Visual-Analog Scale (VAS; 0-10 points). Patients marked the intensity of symptoms with a vertical line on a 10-cm segment, with the left end described as "no symptoms at all" and the right end described as "insufferable symptoms". Each evaluation was marked by the patient on a separate evaluation form. For further analysis, data were treated as parametric. The overall satisfaction derived from the therapy was assessed with the 4-point Verbal Rating Scale (VRS; 0 – completely dissatisfied from treatment, 1 – rather dissatisfied, 2 – rather satisfied, 3 – completely satisfied).

The analysis of costs was based on the mean number of tablets taken during the study period. Number of pills taken was reported by each patient in an Individual Patient's Investigation-Book, which was checked during the follow-up visits. The cost of the least expensive pack of medication was taken into consideration.

The sample size was estimated based on the principle of detecting a 30% difference in the intensity of symptoms, overall satisfaction rate or costs of treatment (meaning clinically relevant difference), with 80% probability at $P<0.05$. A commercially available statistical package (STATISTICA; Stat-Soft, Cracow, Poland) was used for calculations of data entered onto a dedicated spreadsheet (Microsoft Excel 2002; Microsoft Corporation, San Jose, CA, USA). Normally distributed continuous data were analyzed using Student's t-test. Categorical data were analyzed using the χ^2-test or Fisher's exact test (F-test) where appropriate. A P value of less than 0.05 was considered statistically significant. Data are presented as mean values ±SD, and percentages (%).

Results

The investigation included 65 patients with non-erosive gastric reflux diagnosed based on typical symptoms (heartburn, belching, regurgitation) and endoscopy. Of these 65 patients, sixty who responded to initial four-week lansoprazole therapy were randomized to three groups equal in number. In the remaining five individuals, the initial lansoprazole therapy failed to alleviate the complaints and they were found not to be eligible for the stage 2 of the study and excluded. *Tab. 1* presents the characteristics of particular patient groups. Prior to therapy, the mean intensity of complaints in all the groups was 2.8±1.0 points on the VAS scale; following the preliminary therapy, the intensity dropped to the mean value of 0.4±0.5 points. The mean intensity of complaints in particular groups in the course of follow-up is presented in *Tab. 2*. No significant differences were noted between Group 1 and 2. In Group 1, the patients took the mean number of 0.3±0.3 PPI capsules per day. On the other hand, intermittent therapy (Group 3) was significantly less effective as compared to "on-demand" therapy (Group 1) after 6 and 12 months of treatment ($p<0.05$), as well as in comparison to daily therapy (Group 2) after 3, 6 and 12 months ($p<0.05$). Throughout the one-year follow-up, 90% of Group 1 patients were satisfied with their therapy; the satisfaction rate in Group 2 reached 95% and in Group 3, it was 85% (*Tab. 3*).

Table 1. **Characteristics of the patients with NERD (n=20 in each group). There were no significant differences between the groups (* F-test and # χ^2-test)**

Variable	Group 1 "On-demand" treatment	Group 2 Daily treatment	Group 3 Intermittent treatment
Mean age (years) *	49±12	48±11	48±13
Males #	10 (50%)	9 (45%)	11 (55%)
Disease duration # <1 year/>1 year (n)	12/8	13/7	11/9
Smoking #	4 (20%)	5 (25%)	3 (15%)
Alcohol intake #	1 (5%)	0	0

Table 2. **Mean intensity of complaints on the VAS scale depending on the type of therapy. Significant differences (F-test, $p<0.05$) were found between group 1 vs 3 after 6 and 12 months of therapy while between group 2 vs 3 after 3, 6 and 12 months of maintenance therapy, respectively**

	Group 1 "On-demand" treatment	Group 2 Daily treatment	Group 3 Intermittent treatment
Baseline	2.75±1.0	2.95±1.0	2.85±0.9
After 1 month	0.4±0.5	0.5±0.4	0.3±0.5
After 3 months	0.85±0.6	0.65±0.7	1.1±0.6
After 6 months	1.0±0.8	0.65±0.7	1.55±0.7
After 12 months	1.1±0.9	0.5±0.3	1.65±0.8

Table 3. **An overall satisfaction from treatment assessed on the VRS (mean ± SD) and the percentage of patients completely satisfied (% CS) with treatment depending on the type of therapy. There were no significant differences between the groups (* F-test and # χ^2-test)**

	Group 1 "On-demand" treatment		Group 2 Daily treatment		Group 3 Intermittent treatment	
	VRS*	% CS#	VRS*	% CS#	VRS*	% CS#
After 3 months	2.85±0.48	90%	3±0	100%	2.85±0.48	90%
After 6 months	2.9±0.3	90%	2.95±0.22	95%	2.8±0.52	85%
After 12 months	2.9±0.3	90%	2.95±0.22	95%	2.75±0.63	85%

The assessment of therapy costs demonstrated the daily regime to be the most expensive, with the mean expenditure of PLN 151.6 per patient. "On-demand" therapy was cheaper by approximately 30%, with the mean cost of PLN 110.2 per person, and the mean cost of intermittent therapy was lower by approximately 55%, amounting to PLN 68.9 per patient.

Discussion

In patients with GERD, both in initial treatment and in maintenance therapy, proton pump inhibitors are the medication of choice [12]. In recent years, several pharmacological treatment protocols for long-term therapy have been developed, including "on-demand" therapy, daily therapy with low PPI doses, and intermittent therapy, consisting in PPI administration over several weeks in case of recurrent symptoms [4,5]. In numerous investigations completed to-date, the effectiveness to the above-mentioned therapeutic protocols has been demonstrated as compared to placebo [6,13-15]. Nevertheless, no study has been yet conducted that would assess treatment efficacy depending on the degree of reflux symptom intensity. Although there is no correlation between symptom intensity and the intensity of inflammatory lesions involving the esophagus, nevertheless, the severity of the complaints may affect the therapeutic requirements of patients with GERD. This has led the present authors to attempt a pilot comparison of the efficacy and costs of employing the above treatment protocols in maintenance therapy in patients with NERD who report mild complaints.

The investigation has confirmed the high effectiveness of both daily therapy with low PPI doses and "on-demand" therapy. In the latter group, the patients took a PPI capsule every third day on the average, what most likely resulted from their taking the medication not only when they actually experienced reflux-associated symptoms, but also as a "preventive" measure. Nevertheless, the patients highly valued this therapeutic method, emphasizing their ability to individually match taking the medication to their personal needs. Thus, "on-demand" therapy was found to be significantly more cost-effective than daily treatment as it was both as high effective and 30% cheaper (significant difference) than daily treatment. On the other hand, intermittent therapy was characterized by a significantly lower efficacy rate (than both daily treatment and "on-demand" therapy) observed as early as within the initial six months of follow-up, yet, nevertheless, it was also well appreciated by the patients in terms of general satisfaction non-significantly different from other analyzed therapeutic models. This therapeutic model was a source of dissatisfaction for those individuals,

who experienced recurrent disease within a very short time. For others, the overall satisfaction rate was not dependant on the maintenance therapy model, as patients suffered from symptoms of mild intensity, not limiting their daily activity, and achieving a longer time between the relapses was a satisfactory outcome for most of them.

The investigation also confirmed the economic benefits resulting from employing "on-demand" therapy and intermittent therapy, similarly as it was demonstrated by other authors [8,9,16,17].

Summing up, the present authors believe that in long-term treatment of patients with NERD characterized by mild complaints, both "on-demand" therapy and intermittent therapy may be beneficial in view of their effectiveness and economic advantages. However, due to a pilot character of this study further investigation based on a larger number of patients is necessary to confirm the clinical value of cheaper models of maintenance therapy which could be then recommended as more cost-effective than much more expensive daily treatment.

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Elevated concentration of the chemokine CCL3 (MIP-1α) in cerebrospinal fluid and serum of patients with tick borne encephalitis

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Abstract

Purpose: Chemokines, including a chemoattractant for mononuclear cells CCL3 (MIP-1α), are responsible for attracting leukocytes into central nervous system (CNS) and cerebrospinal fluid (CSF) in meningitis and encephalomeningitis. We investigated the possibility of the involvement of CCL3 in tick-borne encephalitis (TBE) pathogenesis.

Material and methods: We studied 26 patients with TBE; 13 with meningitis (group I) and 13 with encephalomeningits (group II). Control group included 11 patients without infectious disease of the CNS. CCL3 concentration was measured by ELISA in serum and CSF on admission (examination 1) and after 2 weeks (examination 2) in TBE patients and once in controls.

Results: In all control samples CCL3 concentration was below detection limit. In TBE, CCL3 serum concentration was: in group I – 10.1±4.1 (mean ±SD, ng/ml) in examination 1 and 12.4±4.8 in examination 2, and in group II – 12.5±3.9 and 13.5±4.8, respectively. In CSF, CCL3 was detected: in group I in 5 patients in examination 1 (178±236 pg/ml) and 11 in examination 2 (457±215), in group II – in 8 (357±311) and 7 patients (326±330), respectively. There were no differences between group I and II. The comparison of CCL3 concentration gradient with albumin gradient between serum and CSF supported the possibility of intrathecal synthesis of CCL3.

Conclusions: 1) Synthesis of CCL3, perhaps including intrathecal synthesis, is increased in TBE. 2) CCL3 concentration was much lower in CSF than in serum of the TBE patients, which argues against its significant role as chemoattractant in this condition.

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Introduction

Tick-borne encephalitis (TBE) is an acute infectious disease caused by a virus of Flaviviridae family, endemic in the north-east of Poland [1,2]. TBE virus is transmitted to man from animal reservoir through a bite by Ixodes sp. tick [3]. Two hundred sixty two cases of TBE were diagnosed in Poland in 2004 (morbidity 0.69/100 000), including 218 cases in the North-East regions of the country [4]. The infection may be asymptomatic or present only with mild, unspecific symptoms, and in such cases it usually remains unrecognized. If the central nervous system (CNS) becomes involved, the clinical course may range from relatively mild meningitis through meningoencephalitis to severe cases with spinal cord and/or spinal nerve roots involvement [2,5]. TBE may be a life-threatening disease and cause permanent neurological deficits, sometimes resulting in a serious disability [2,3,5,6].

A local inflammatory response in TBE manifests itself with perivascular infiltrates within the CNS and pleocytosis of the cerebrospinal fluid (CSF), both with a predominance of mononuclear cells [3,5,7,8]. Both animal models and some clinical observations suggest that an excessive and inadequate local inflammatory reaction and cellular response may be a factor contributing to a CNS damage in course of a flavivirus infection [7,9,10-12]. In fact, high CSF pleocytosis has been linked to a more severe course of TBE, less effective humoral response against TBE virus and higher risk of permanent neurological sequele [2,7]. Studies on mice infected with West Nile virus, which is another neurotropic member of Flaviviridae family, suggest that especially CD8+ (cytotoxic) lymphocytes infiltrating CNS may directly contribute to the CNS damage in this setting [12].

Chemokines are a large group of proinflammatory cytokines exerting chemotactic effect on leukocytes. In inflammation

leukocyte migration to an inflammatory focus is driven by a chemokine concentration gradient. Specific chemokines differ with regard to they target leukocyte populations [13]. In CNS infections of both viral and bacterial ethiology chemokines create chemotactic gradient responsible for leukocyte migration into CNS and CSF, which is reflected by their increased concentrations in CSF [14-17]. Chemokine CCL3, also referred to as macrophage inflammatory protein 1-alfa (MIP-1α) is a chemoattractant for mononuclear cells: monocytes and activated T lymphocytes, especially for CD8+ lymphocytes, with some activity towards CD4+ cells as well [18,19]. CCL3 seems to be associated with a Th1 response, which typically dominates in infections by intracellular pathogens and is essential in the development of cellular immunity [18-20]. This characteristics suggests that this chemokine might be involved in the pathogenesis of TBE, contributing to the CSF mononuclear pleocytosis and formation of cellular infiltrates within CNS. Especially, its predominance towards cytotoxic lymphocytes makes it a factor potentially involved in CNS destruction in severe cases of TBE.

The purpose of the study was to evaluate if CCL3 may play a role in the pathogenesis of TBE. With that aim, CCL3 concentration was measured in CSF and serum of patients with meningitis or encephalomeningitis caused by TBE virus, both in the early stage of the disease (directly after hospitalization) and in the convalescent phase (after 2 weeks), and its correlation with the inflammatory parameters of the CSF was analyzed.

Material and methods

The study group included 26 patients with TBE (4 women and 22 men) aged from 20 to 67 years ($\bar{x} \pm SD$ – 40.8 ± 12.9 years) hospitalized in years 2001-2004 in the Department of Infectious Diseases and Neuroinfections of the Medical University of Białystok. A preliminary diagnosis of TBE was established on the basis of a clinical symptoms of meningitis or encephalomeningitis and an epidemiological history (a tick bite or exposure to ticks in the endemic area during a previous month). In all the patients the diagnostic lumbar puncture revealed CSF with inflammatory abnormalities suggesting lymphocytic meningitis. In one patient included in the study, no abnormalities were found in the CSF from the first lumbar puncture, probably due to the early stage of the infection, but typical changes appeared in the CSF obtained from the following puncture 9 days later. In all the patients, the diagnosis of TBE was confirmed by the detection of antibodies against TBE virus in serum and/or CSF samples by an enzyme-linked immunosorbent assay (ELISA) with a diagnostic kit from Virion/Serion GmbH (Würzburg, Germany).

Meningoencephalitis was diagnosed in patients with neurological abnormalities found in physical examination. Diagnosis of meningitis was established in the remaining patients. Altogether, 13 patients had meningitis (group I) and another 13 meningoencephalitis (group II); of the latter 3 presented with the neurological lesions suggestive of myelitis or spinal radiculitis as well. In meningoencephalitis group Babinski and/or Oppenheim signs were observed in 10 patients, cerebellar symptoms – in 7 patients, limb paresis – in 3 and tremor – in 1.

A control group consisted of 11 patients, in whom diagnostic lumbar puncture excluded meningitis and no antibodies against TBE virus were detected.

All patients gave consent to participate in the study and its scheme was approved by the Ethics Committee of the Medical University of Białystok.

The blood and CSF samples were obtained together with the samples taken for routine laboratory examinations. The samples from TBE patients were collected twice: in the first 24 hours of hospitalization (examination 1) and at the time of the check-up lumbar puncture performed in the convalescent phase of the disease (examination 2), after a mean period of 15.7 days (9-20 days, ±4.3). The blood samples were clotted and than centrifuged within 15 minutes after collection, after which the serum was frozen till further examinations. The CSF samples were stored at 5°C for ≤24 hours and afterwards frozen. The samples were stored in the freezing chamber at the temperature of -80°C.

Pleocytosis and total protein concentration in CSF and albumin concentration in serum were determined by standard laboratory techniques. Albumin concentration in CSF was assayed by the nephelometric method using Turbox system.

CCL3 concentration was determined in serum and CSF samples by the EIA method with the kit from PromoCell GmbH (Heidelberg, Germany), strictly following the manufacturer's instructions. The serum samples were diluted fourfold, and the CSF – twofold, according to the manufacturer instructions. The sensitivity of the test was 195 pg per ml of the dilution, which with the dilutions applied made it possible to determine concentrations ≥390 pg/ml is CSF and ≥780 pg/ml in the serum. Absorbency was read by ELX 800 reader (Biotec Instruments Inc.) at the 492 nm wavelength. CCL3 concentration was calculated from absorbency with the standard curve drawn according to the manufacturer's information. Concentration below the detection limit were considered 0. The values read from the curve were than multiplied by 2 for the CSF and by 4 for the serum.

The ratio of albumin concentrations in CSF and serum (albumin index) was used to assess a dysfunction of the blood/ /CSF barrier.

To correct for the inflow of CCL3 into the CSF from the serum and to asses if CCL3 synthesis could occur intrathecally, the following ratio of concentrations: (CCL3 in CSF/ CCL3 in the serum)/(albumin in the CSF/albumin in the serum) ("CCL3 index") was used. With this formula, the value ≥1 may be regarded as suggestive of intrathecal synthesis [21].

Statistical analysis was performed using SSST software. Mann-Whitney's test was used to compare the groups. The t-test for dependent samples was used to compare concentrations in the serum and CSF, and in the examinations 1 and 2. Correlations between the parameters were evaluated by chi-square test. The $p<0.05$ was considered statistically significant.

Results

CSF inflammatory parameters

Data on CSF cytosis in TBE patients with statistical interpretation are presented in *Tab. 1*. Total protein concentration

Table 1. **CSF cytosis in patients with tick-borne encephalitis during the first 24 hours of hospitalization (ex. 1) and after 2 weeks (ex. 2). I – patients with meningitis (n=13), II – patients with encephalomeningitis (n=13), I + II – all patients with TBE virus infection (n=26). The values are expressed as cells/mm^3**

	total			mononuclear		polynuclear	
	min-max	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
I – ex.1	2-196	103	68	76*	44	44	21
I – ex. 2[a]	11-150	47 &	41	-	-	-	-
II – ex.1	20-373	170	125	156*	101	59	68
II – ex. 2[a]	4-112	39 &	30	-	-	-	-
I + II – ex. 1	2 - 373[b]	153	105	114	86	50	48
I + II – ex. 2[a]	4 - 150[b]	41 &	31	-	-	-	-

min-max – range of values observed; x – mean; SD – standard deviation; * – significant difference between groups I and II ($p<0.05$); & – significant decrease in comparison with examination 1; [a] – exact numbers of mono- and polynuclear cells were not counted in large fraction of patients due to small general pleocytosis; as a rule CSF smear in examination 2 revealed exclusively or almost exclusively mononuclear cells; [b] – in all patients cytosis was above 10/mm^3 in at least one examination (ex. 1 or ex. 2), allowing for the diagnosis of meningitis/meningoencephalitis

Table 2. **CCL3 concentrations in the serum and CSF of patients with TBE virus infection during the first 24 hours of hospitalization (ex. 1) and after 2 weeks (ex. 2). I – group of patients with meningitis (n = 13), II – group of patients with encephalomeningitis (n = 13), I + II – all patients with TBE virus infection together (n = 26). In controls (n = 11), all CCL3 concentrations in the serum and CSF were below the detection limit. The values are expressed in pg/ml**

	CSF				serum			
	n (%)	min-max	$\bar{x}$	SD	n	min-max	$\bar{x}$	SD
I – ex.1	5/13 (38%)	0 – 542.2	178#	236	13/13 (100%)	6808-18156	10104&	4170
I – ex. 2	11/13 (85%)	0 – 647.2	457#	215	13/13 (100%)	6700-19232	12405&	4751
II – ex.1	8/13 (62%)	0 – 861	357	311	13/13 (100%)	7800-20188	12518&	3906
II – ex. 2	7/13 (54%)	0 – 897.4	326	330	13/13 (100%)	8872-23260	13521&	4826
I + II – ex. 1	13/26 (50%)	0 – 861	267	286	26/26 (100%)	6808-20188	11421&	4188
I + II – ex. 2	18/26 (69%)	0 – 897.4	391	281	26/26 (100%)	6700-23260	12963&	4637

min-max – range of values observed; x – mean; SD – standard deviation; n (%) – number and percentage of patients with detectable CCL3 concentration; # – statistically significant difference between examination 1 and 2, $p<0.01$; & – concentration higher in the serum than simultaneously in the CSF, $p\leq0.001$

was moderately increased in CSF of patients with TBE and did not differ between group I and II (data not shown).

In group I (examination 1) total protein concentration in the CSF correlated with total (0.60, $p<0.05$) and mononuclear (0.62, $p<0.05$) pleocytosis. In examination 2 the correlation remained for total pleocytosis (0.66, $p<0.05$). This correlation was not observed in group II.

Blood/CSF barrier dysfunction

The albumin concentration in CSF could be measured in only about 2/3 of the collected samples, due to the small quantity of CSF available. In that samples, the mean albumin concentration did not differ between group I and II. In both TBE groups it was increased more than fourfold in examination 1 ($p<0.01$) and more than twofold in examination 2 ($p<0.05$) in comparison with controls. A decrease in albumin concentration in examination 2 was statistically significant in both group I and II ($p<0.05$). The mean albumin concentration in serum showed no difference between groups or between examination 1 and 2.

In examination 1, the mean albumin index was 0.031 ± 0.020, and in examination 2 – 0.014 ± 0.010 (calculated for groups I and II taken together) and was significantly higher compared to controls (0.006 ± 0.004), confirming an increased permeability of the blood /CSF barrier.

CCL3 concentration in CSF and serum

The mean concentrations of CCL3 in serum and CSF together with statistical interpretation are shown in *Tab. 2*. In TBE patients, CCL3 concentration exceeded the detection limit in all serum samples, whereas in CSF it was detectable in only slightly over 50% of all the samples. In controls, all concentrations of CCL3 remained below the detection limit. Of note, mean concentrations of CCL3 were several fold higher in serum than in CSF.

CCL3 concentration in CSF was not correlated with pleocytosis. Correlation between CCL3 and protein concentration in CSF was found only in examination 1 in group I (correlation 0.59, $p<0.05$).

CCL3 index

Complete data needed to calculate "CCL3 index" (including albumin in CSF and detectable CCL3 concentration in CSF) were available only in individual patients: in group I in two patients in examination 1 and in five patients in examination 2, and in group II – in three patients in examination 1 and two patients in examination 2. Only in one case (in group I, examination 1) the value so calculated was lower than 1 and equaled 0.58. The remaining values of "CCL3 index" ranged from 1.04 to 33.25 and showed a tendency to increase in examination 2

(median in examination 1 – 1.42, in examination 2 – 3.14), however, due to the small number of the cases, no further statistical analysis could be performed.

Discussion

In this study, the increased concentration of CCL3 was found both in serum and CSF of patients with TBE. The possibility of CCL3 synthesis within CNS has been previously confirmed by *in vitro* studies [22,23]. Human microglial cells are capable of producing minimal CCL3 quantities in the non-stimulated culture and of a rapid (in a few hours time) and prolonged (maintained for 48 hours) increase in its synthesis under stimulation with proinflammatory factors, such as lipopolisacharid (LPS), tumor necrosis factor alpha (TNF-α) and interleukin 1 (Il-1) [22]. *In vivo*, CCL3 has been detected, although in rather low and variable concentrations, in the CSF of patients with viral CNS infections. In patients with viral meningoencephalitis studied by Lahrtz et al., CCL3 was present in the concentration exceeding detection limit of 300 pg/ml in 22% of the CSF samples [24]. Inaba et al. discovered CCL3 in mean concentration of 161 pg/ml in CSF of 17 of 22 patients with viral meningoencephalitis [15]. Rosler et. al. observed a markedly increased concentration of CCL3 in the CSF of three patients in early phase of encephalitis caused by herpes simplex virus (HSV) [25]. Despite a marked increase, CCL3 concentration correlated neither with inflammatory parameters of the CSF nor with patients' clinical condition [25]. Data on CCL3 presence in the CSF of patients with CNS disorders in course of HIV infection are contradictory [26,27]. However, Letendre et al. found CSF CCL3 concentrations up to few hundred pg/ml in this population of patients, which correlated with clinical findings. Interestingly, higher concentrations of CCL3 seemed to have a neuroprotective effect, whereas detectable but low concentrations correlated with the unfavorable course of CNS involvement and development of dementia [27].

There is little data on the role of chemokines in the pathogenesis of TBE and CCL3 in particular has not been investigated so far. However, increased concentrations of two chemokines: CCL2 (monocyte chemotactic protein-1, MCP-1) and CCL5 (RANTES), have been detected in CSF in TBE patients [28,29]. More complex data have been gathered on the role of chemokines in Japanese encephalitis (JE), condition caused by a flavivirus closely related to TBE virus. Singh et al. found a several fold increased concentration of interleukin 8 (Il-8, CXCL8), a potent proinflammatory agent and chemoattractant for neutrophils and activated T lymphocytes, in CSF and serum of patients with JE. Il-8 concentration was higher in patients with a more sever disease and decreased more slowly in patients with prolonged symptoms [30]. Winter et al., studying a large group of patients with JE, found Il-8 and CCL5 in CSF in the increased concentrations not correlating with concentration in serum, which argues in favor of their intrathecal synthesis. CCL5 concentration in the CSF correlated with the total pleocytosis, which supports the role of this chemokine as a chemoattractant. There was a correlation between high concentrations of proinflammatory cytokines, including Il-8 in the CSF and CCL5 in the serum, with an unfavorable clinical course of the JE and weaker humoral response. It remains unclear, however, if high chemokine concentrations in JE are themselves a factor contributing to the CNS damage or just a sign of a massive CNS involvement and poor clinical outcome [9].

In our study, CCL3 concentration was much lower in CSF than simultaneously in serum. An increase in total protein and albumin concentrations in the CSF and increase of the albumin index suggest the dysfunction and increased permeability of the blood/CSF barrier in the TBE patients, more pronounced in examination 1 and partially resolved in examination 2. Because of that it can be speculated that increased concentration of CCL3 in CSF was caused not by a local intrathecal synthesis but rather by permeation from serum via the blood/CSF barrier. However, we observed that an increased concentration of CCL3 was maintained (or even further elevated in patients with meningitis) in examination 2, when the blood/CSF barrier dysfunction tended to resolve. This observation is supported by the assessment of 'CCL3 index', although it was calculated only in few patients and provided the results which highly varied between individual patients. The high values of this index, tending to increase in examination 2, argue in favor of the intrathecal synthesis of CCL3.

CCL3 concentration in the CSF correlated neither with its pleocytosis (both total and a quantity of mononuclear cells) nor with the clinical severity of the disease. Taken these into consideration, it seems not likely that a gradient of this chemokine is a main factor controlling the leukocyte inflow to the CSF and some other factors involved in this process should be considered, including other chemokines. Michałowska-Wender et al. detected an increased concentration of monocyte chemotactic protein (MCP-1) in the CSF of patients with TBE but, similarly to CCL3 in our study, MCP-1 concentration did not correlate with pleocytosis [28]. The mononuclear cells present in CSF at the early stage of TBE are mainly T CD4+ lymphocytes and less numerous T CD8+ cells, with a scarce number of NK cells and B lymphocytes, which corresponds fairly well with the spectrum of activity of two chemokines related structurally to CCL3: CCL4 (MIP-1β) and CCL5 [8,18,19,31]. Concentrations of CCL4 have not been studied in TBE. In our previous study, we observed increased CCL5 concentration in the CSF of patients with TBE, but we failed to document its intrathecal synthesis and its correlation with pleocytosis [29].

As observed by Holub et al., a number of T cells and their particular subpopulations in the CSF of TBE patients correlate well with the total protein concentration, which led the authors to imply that lymphocyte migration in this patients is a rather unselective and passive process, dependent more on a dysfunction of the blood/CSF barrier than on the activity of specific mediators and chemoattractants [8]. This could possibly explain the lack of correlation between CSF cytosis and concentrations of chemoattractants studied so far. In our study, the total and mononuclear pleocytosis correlated with the CSF protein concentration in patients with meningitis. In meningoencephalitis group, however, mononuclear pleocytosis was not only substantially higher, but also did not correlate with protein level. This difference, if confirmed, might suggest a more selective and specific recruitment of lymphocytes into the CNS in patients

with a more severe form of the infection; however, factors responsible for this recruitment remain to be elucidated.

The results presented above suggest some level of intrathecal synthesis of CCL3 in TBE, but do not argue convincingly in favor of its significant role as a factor responsible for CSF pleocytosis. Still, certain pathogenetic role of CCL3 cannot be excluded. According to its spectrum of activity, CCL3 could contribute mainly to the inflow of CD8+ lymphocytes, which are less numerous than CD4+ cells in the CSF of TBE patients. Of note, data from animal model (mice infected with West Nile Virus) suggest CD8+ cells may be essential for both clearing the flavivirus infection of CNS and accompanying tissue damage, but it remains unknown whether this observations can be extended human infection [12]. The role of CCL3 and other chemokines in the pathogenesis of TBE requires further investigations.

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The emotional component of the attitude of the physician in situations of obstetric failure

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Abstract

Purpose: The research aim is to study the working attitude of a physician towards his patient with obstetric failures in the emotional component aspect.

Material and methods: A sample of 164 gynecological doctors was encompassed by the study. The physicians were mainly interviewed during various types of training courses, specialist conventions and during personal meetings. A 44-question anonymous questionnaire was directed at gynecologists. The question was closed. The survey used for the research (in "ex post facto" procedure) matches quantity and quality elements.

Results: Concerning the emotional aspect referred to the most difficult in the physician – patient relation: 18% of the respondents stated they had positive feelings towards the patient,16% had self-centred feelings and 1% had negative feelings towards the patient. Concerned the feelings of the doctor when the patient and her husband are in a situation of obstetric failure: 49% shared positive feelings in experiencing obstetric failure in patients, 38% concentrated on themselves and their own feelings and 4% gave a decided negative reply. Physicians' attitudes were measured in relation to the death of a patient: 52% related that experience very personally to themselves, 4% of the physicians referred to the death of their patient with a sense of great sorrow and 1% were negatively trying to put the blame on the deceased patient.

Conclusions: The most emotionally difficult obstetric failure in the doctor – patient relation was the death of a prenatal child; the most effective reaction to the sorrow of a mother after the loss of her child was support and bringing relief to the patient; 38% of gynecologists have not answered the question because of luck of such experience and because of the too difficult trauma experience.

Key words: emotional component, physicians attitude, obstetric failure.

Introduction

The review of the literature as well as gynecological practice inspired to undertake the research aiming to emphasize the doctor's conduct and looking for the appropriate one behavior, which help the patent to recover quickly and to get back to normal life in the society. This will bring the patient to the state of homeostasis of her attitude and help her to realize her procreative plans [1-3].

The patient – doctor relationship is developing also on the psychological basis. There is an exchange of information and emotions, the parts interact between themselves.

Knowledge about doctor – patient relationship after obstetrical failure is very important and needed for communicational skills in order to make it constructional for both parts.

The confrontation of the feeling of a joyful anticipation of the child and the frustration connected with an obstetric failure influences the physician – patient relation after the loss of a child. An obstetric failure and the subsequent separation process with the child present in the parents as well as the doctors in whom it is significantly milder, is a long-term process the end of which is impossible to foresee [4-6].

The following obstetric failure have been considered and examined as obstetric failures: spontaneous miscarriage, premature birth, intrauterine death, postpartum child death, giving birth to a child with developmental defects, sterility and artificial abortion.

The research aim is to study the working attitude of a physician towards his patient with obstetric failures in the emotional component aspect.

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Material and methods

A sample of 164 gynecological doctors was encompassed by the study. The physicians were mainly interviewed during various types of training courses, specialist conventions and during personal meetings. The study was conducted in the years 2001--2004. The doctors comes from different parts of Poland. 20% of them comes from small towns and villages, 28% from towns up to 100 thousand inhabitants and 52% works in the cities with number of inhabitants bigger then 100 thousand. 20% of gynaecologists works in clinics, 53% in the hospitals, 7% in outpatient clinics and remaining 20% works in two or three places at the same time. The doctors age ranges from 27 to 59. 61% of them were male and 39% were female. 83% among surveyed doctors were II degree specialists and 17% were during the specialization courses. Their seniority ranges from 5 to 33 years.

A 44-question anonymous questionnaire was directed at gynecologists. The questions concerned their personal details such as age, sex, work experience, current place of work and their approach to faith. The remaining questions concerned the problem of the attitudes of physicians towards their patients after an obstetric failure. Doctor's attitude comprise three components: cognitive, behavioral and emotional. On the emotional basis the doctor were asked about most difficult obstetrical failure.

The question was closed. Then the doctors were asked about their feelings in the situation of the patient's death and their experience of their interaction with the patients and the patients' families while in obstetrical failure. This question was opened and evaluated in the judicial method. The survey used for the research (in "ex post facto" procedure) matches quantity and quality elements. This method was used based on the attitude theory. The method used in the research was an independent empirical procedure.

Physicians' attitudes were studied in their emotional aspect.

The emotional component of an attitude includes:

a) positive feelings towards the patient:
– compassion
– the desire to help
– sharing one's own experiences

b) negative:
– indifference
– lack of identification with the patient
– disillusion with the patient

c) feelings directed at oneself:
– dejection
– sense of guilt, failure
– case analysis
– difficulties in assuming the appropriate attitude.

Results

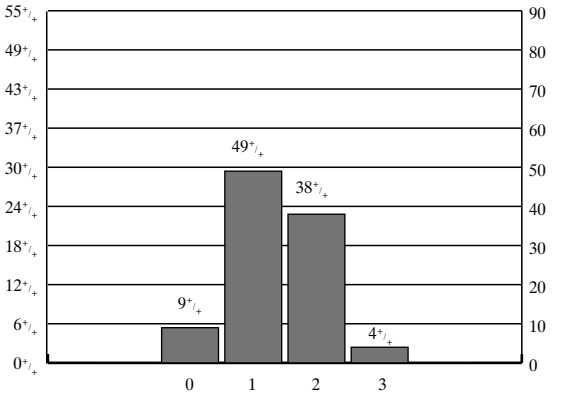
The results presented below concern the attitudes of gynecologists towards their patients that had an obstetric failure (emotional component).

Emotional components of the attitude of the doctor towards the patient with an obstetric failure.

Table 1. **The most difficult obstetric failure in terms of emotions in the doctor – patient relation**

Types of physicians' feelings	Amount	%
Positive towards the patient	29	19
Negative towards the patient	2	1
Self-centred	26	16
Ambivalent feelings	12	7
No response	95	57
Total	164	100

Figure 1. **Doctors' feelings directed towards the patient in an obstetric failure situation**



0 – no previous experience; 1 – positive feelings directed at the patient; 2 – self-centred physicians' feelings; 3 – negative feelings towards the patient

The question concerning the emotional aspect referred to the most difficult in the physician – patient relation chosen obstetric failure of a patient. 57% failed to give a reply in this issue because of luck of personal experience and emotional problems and 19% of the respondents stated they had positive feelings towards the patient. 16% had self-centred feelings and 1% had negative feelings towards the patient. 7% of the respondents had mixed feelings, self-centred and positive or negative feelings towards the patient (*Tab. 1*).

The next question concerned the feelings of the doctor when the patient and her husband are in a situation of obstetric failure.

9% of gynecologists did not provide an answer, 49% shared positive feelings in experiencing obstetric failure in patients, 38% concentrated on themselves and their own feelings and 4% gave a decided negative reply in relation to those experiencing failure of their wife or the husband of the patient (*Fig. 1*).

Physicians' attitudes were measured in relation to the death of a patient and their feelings connected with death. 38% of the respondents failed to give a reply or did not have any such experiences and as many as 52% related that experience very personally to themselves; 4% of the physicians referred to the death of their patient with a sense of great sorrow and 1% were negatively trying to put the blame on the deceased patient whereas 5% had dominantly mixed feelings (*Tab. 2*).

Table 2. **Physicians' feelings in the situation of their patient's death**

Physicians' feelings	Amount	%
Positive towards the patient	6	4
Negative towards the patient	3	1
Self-centred	84	52
Ambivalent feelings	9	5
No response	62	38
Total	164	100

Discussion

The emotional aspect of communication with the patient simultaneously revealing the attitude of the physician to their patient with an obstetric failure was provided by questions concerning the feelings of physicians in a situation of obstetric failure in their patients and in the situation of the death of their patients.

In a situation of a patient and her husband experiencing obstetric failure, the doctors experienced the following: 1% had a strongly positive approach and interest in the patient. As many as 38% of the respondents were concentrating on themselves and their own feelings, failing to notice the patient and her relatives; 4% of gynecologists had an attitude of claims towards the patient and 9% of the respondents did not give any answer. The physicians stated that they have not undergone any training or course on the stages of mourning after the loss of a child. Heiman [7] has written 'The Touching Hearts Program at the University of Iowa' – for medical personnel facilitating the support provided to parents and their immediate environment after the loss of their child. The Programme, among others, encourages seeing the baby, spending private time with their baby, choosing a name for their baby, meeting the hospital chaplain, and offers help in organising the funeral as well as many other aspects. The medical personnel are instructed within the Programme on ways of informing of the loss of a child and how to experience the loss of their child as well as offering help to others after the death of their child.

Schaap [4] encourages physicians to identify with 'risk pairs' requiring special or additional help as a lack of earlier medical intervention in those patients and their families may cause irreversible affects, impossible to heal even with the passage of time. The author proposes that physicians encourage patients to express their grief, to identify the feelings they are experiencing.

Wiener [8] has stressed that parents have a great desire to be comforted and supported and they have a need of experiencing the doctor's approachability.

The emotional aspect of physicians' attitudes is reflected in the feelings of gynecologists in the situation of the death of their patient. It is interesting that 38% of the respondents did not provide an answer to the given problem – partly due to a lack of personal experience. There were, however, gynecologists whose perceptive powers were incapable of encompassing the ordeal – 'This cannot be put into words; very personal experience' or 'thankfully I have never gone thorough a similar situation and I hope I never will'. In the situation of the death of a patient as many as 52% of gynecologists were self-centred and were analysing whether everything was carried out. Their experiences were accompanied by dejection, a sense of disappointment, emptiness. 4% of the respondents felt a sense of relief in connection with the death of their patient if it entailed an end to the suffering of their patient. 6% of gynecologists negatively assessed the event of death by reacting with anger, indifference and a sense of failure as well as a lack of identification with the patient.

The event of death, particularly of their own patients that were under the medical care of the physician, gives rise to deep repercussion, reflection and compels them to verify the medical attitudes or analyses into algorithms for action. This is also confirmed by studies conducted by Lewis [9,10,11] and Speck [12]. A special situation is the death of a patient in the perinatal stage or the death of a mother and her child [15].

Conclusions

The following conclusions were drawn from the performed research:

1. The most emotionally difficult obstetric failure in the doctor – patient relation was the death of a prenatal child.
2. The most effective reaction to the sorrow of a mother after the loss of her child was support and bringing relief to the patient.
3. In the situation of patient's death – 38% of gynecologists have not answered the question because of luck of such experience and because of the too difficult trauma experience. 52% were concentrated mostly on themselves and the analyze of the case, 6% were frustrated and expressed their defeat, 4% felt relief if the patient's death stopped her suffer.

The emotional aspect of the physician – patient attitude after an obstetric failure presented and described herein, brings new elements both cognitive and pragmatic into general doctor – patient communication.

In practice, this will enable a physician to gain a better understanding of the psychological situation of a patient after an obstetric failure and at the same time prepare her for her next pregnancy.

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Influence of a physical exertion on the workers' health state

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Abstract

Purpose: The health state of the workers employed at hard and very hard workplaces (on the ground of an energy expenditure measurement) has been presented in this report.

Material and methods: The morbidity of these workers was compared with the morbidity rates of two groups: the persons employed at a factory management and the workers performing light and moderate physical work. The number data has been standardized in order to eliminate the influence of an age over the morbidity rate values.

Results: A number of the considerably differences in frequency of the occurrence of each disease among the examined employees groups has been found.

Conclusion: The diseases of the peripheral nervous system and locomotive organs as well as the disturbances of the cerebral circulatory were of more frequent occurrence among the persons performing hard and very hard work.

Key words: epidemiology, physical exertion, health state.

Introduction

The technical progress in many branches of the industry limited considerably a physical effort, particularly its dynamic component. However, not everywhere is possible a total automatization of production. On the other side making the processes of the production more modern causes the efficiency's increase, which is related to growth of its rate, which in turn causes that work is harder.

The physical effort extend, which is connected to a functional equilibrium, i.e. homeostasis, is characteristic of every person. It is a submaximal effort. Efficiency in taking this kind of effort is the bigger the bigger is the efficiency of a respiratory system and a circulatory system. The maximal effort which exceed organism's functional equilibrium leads to progressive changes in organism's reactions [1-3].

Regardless of age, sex and physical ability there is the linear dependence between the increase of oxygen consumption and the effort value. Simultaneously, independently to the mentioned before factors, there is a linear dependence between oxygen consumption and the minute heart volume, and the same between effort value and minute heart volume. Stroke volume reach its maximal level when the oxygen consumption is about 30% of the maximal ability of the oxygen absorption by human's organism. Increasing heart minute volume above this value is mainly achieved by increasing the of heart rate [1].

On the ground of many researches it was settled that 8 hours of work is not to big workload for a worker when the energy expenditure counted for work – day is lower than 30% of the individual maximal organism's possibilities. Crossing over this possibilities leads to the oxygen debt with all consequences to the health [3,4].

Results of many researches point at the meaning of the level of physical efficiency as a protective factor in the pathomechanism of circulatory system diseases development [5-8].

Low back pain is understand as a morbid complex with a different etiology and pathogenesis, which common symptom is low spine pain. In the contemporary society they are not only the serious health problem but also the social and the economic problem. In the work medicine this problem is mainly concerned with people whose work is connected with factors that cause or intensify low back pain. Here can be mentioned: a hard physical work, changing atmospheric conditions, an affected body position during the work, sitting work and also exposure of vibration [9-11].

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Table 1. **Occurrence of the diseases among two workers groups (hard and very hard physical work against mental work) – the age specific and age standardized morbidity**

Diseases categories	Hard manual work		Mental work		Chi²	Differences p<0.05	ASM	
	n	%	n	%			Hard manual work	Mental work
Circulatory system	78	22.3	59	34.3	8.49	s	26.4	30.3
Arterial hypertension	41	11.7	40	23.3	11.52	s	15.9	14.8
Ischemic heart	27	7.7	18	10.5	1.09	ns	9.1	7.8
Disturbances of cerebral circulatory	11	3.2	4	2.3	0.28	ns	4.0	2.0
Respiratory system	28	8.0	11	6.4	0.44	ns	9.5	5.7
Digestive system	51	14.6	45	26.2	10.23	s	17.3	23.2
Chronic gastric and duodenal ulcer	25	7.4	20	11.6	3.05	ns	8.5	10.3
Endocrine glands and metabolic	67	19.2	47	27.3	4.45	s	22.8	24.1
Thyroid gland	26	7.4	14	8.1	0.08	ns	8.8	7.2
Hyperlipidemia	29	8.3	28	16.3	7.51	s	9.8	14.4
Peripheral nervous system	45	12.9	12	7.0	4.14	s	15.3	6.2
Musculo-osseous system	77	22.1	33	19.2	0.57	ns	26.2	17.0
Discopathy	38	10.9	13	7.6	1.45	ns	12.9	6.7
Neurosis	31	8.9	16	9.3	0.02	ns	10.5	8.2
Neoplasms	8	2.3	7	4.1	1.30	ns	2.7	3.6
Employed with diseases – total	218	65.5	117	68.0	0.75	ns	66.6	60.1
Employed – total	349		172		-			

ASM – age standardized morbidity (standard – the general population); s – significant; ns – nonsignificant

The goal of this paper was the estimation the influence of different kinds of work on the health condition of workers employed at one of the ceramic factories.

Material and methods

The examinations were performed in 1020 workers (714 women, 306 men) employed at one of the ceramic factories. They were between 19 and 64 years old (mean 35.8±9.9 years). Most of the workers (848 persons) were employed at physical work, the others persons (172) were working at mental work (management).

The level of the workload was examined on the basis of the amount of the energy expenditure [4,12].

The method of indirect calorimetry [4,13] was used to measure the energetic level during the fundamental works on 99 work places. This method consists of the minute ventilation quantification by the meter of energy expenditure WE – 4, elaborated by Centralny Instytut Ochrony Pracy in Warsaw. Time of every measurement was about 10 minutes. The energy expenditure of the auxiliary work (arrangement, cleaning) and breaks during the work was quantified by the tabular method [4,14]. The researches of energy expenditure were preceded by detailed timing of work shift. The total energy expenditure was presented in net values of the effective professional work, after the deduction of the basal metabolism [4,13]. Calculations were standardized on the weight of 70 kilograms for men and 60 kilograms for women [146].

The workplaces on which total energy expenditure exceeded 1500 kcal (6300 kJ) for men and 1000 kcal (4200 kJ) for women were qualified as a hard and very hard work [12].

Evaluation of the health condition was made on the ground of general and specialistic medical examinations (subjective and objective) and laboratory tests.

The morbidity of workers employed at hard and very hard workplaces was compared with the morbidity rates of two groups: the persons employed at a factory management and the workers performing light and moderate physical work. The number data has been standardized in order to eliminate the influence of an age over the morbidity rate values.

Results

In the *Tab. 1* there were compared frequency of incidence of chronic illnesses among workers employed on work positions connected with hard and very hard physical work with morbidity rates of mental workers (factory management workers). Among the first group of workers (hard and very hard work) the occurrence of peripheral nervous system diseases was observed statistically more frequent ($p<0.05$). It was similar with respiratory system diseases, musculo-osseous system (especially spine) diseases and disturbances of the cerebral circulatory like headache and vertigo, but the differences were non significant. Other circulatory system diseases, digestive system diseases, endocrine glands and metabolism diseases occurred more frequent among mental workers. This relationships are more visible for standardized coefficients.

There were also differences in frequency of occurrence of chronic illnesses among workers performing hard or very hard physical work and workers performing light or moderate physical work (*Tab. 2*). Among the first group (hard and very hard work) some of the circulatory system diseases like ischemic heart disease, disturbances of the cerebral circulatory or spine diseases (discopathy) occurred statistically more frequent ($p<0.05$). Among these workers also occurrence of other musculo-osseous

***Table 2*. Occurrence of the diseases among two workers groups (hard, very hard physical work against light and moderate work) – the age specific and age standardized morbidity**

Diseases categories	Hard manual work		Light and moderate work		Chi^2	Differences $p<0.05$	ASM	
	n	%	n	%			Hard manual work	Light and moderate work
Circulatory system	78	22.3	139	27.9	3.27	ns	26.4	26.6
Arterial hypertension	41	11.7	61	12.2	0.04	ns	15.9	11.6
Ischemic heart	27	7.7	22	4.4	4.18	s	9.1	4.2
Disturbances of cerebral circulatory	11	3.2	4	0.8	6.53	s	4.0	0.7
Respiratory system	28	8.0	47	9.4	0.50	ns	9.5	9.2
Digestive system	51	14.6	77	15.4	0.11	ns	17.3	14.7
Chronic gastric and duodenal ulcer	25	7.4	41	8.2	0.22	ns	8.5	7.8
Endocrine glands and metabolic	67	19.2	103	20.6	0.27	ns	22.8	19.6
Thyroid gland	26	7.4	34	6.8	0.13	ns	8.8	6.5
Hyperlipidemia	29	8.3	26	5.2	3.25	ns	9.8	5.0
Peripheral nervous system	45	12.9	62	12.4	0.04	ns	15.3	11.8
Musculo-osseous system	77	22.1	93	18.6	1.50	ns	26.2	17.7
Discopathy	38	10.9	34	6.8	4.39	s	12.9	6.5
Neurosis	31	8.9	28	5.6	3.39	ns	10.5	5.3
Neoplasms	8	2.3	7	1.4	0.94	ns	2.7	1.3
Employed with diseases – total	218	65.5	299	59.9	0.14	ns	66.6	57.1
Employed – total	349		499		-			

ASM – age standardized morbidity (standard – the general population); s – significant; ns – non significant

system diseases, peripheral nervous system diseases, thyroid diseases, hyperlipidemia, neurosis and tumors were more frequent but differences were statistically non significant ($p<0.05$).

Discussion

Hard physical work and at the same time lack of physical activity like sport and also low physical efficiency are the factors which are conductive to more frequent occurrence of ischemic heart disease [15]. It was find, that percentage of people with risk factors of this disease (obesity, arterial hypertension and others) was the highest among these people who worked hard and did not make a sport and the lowest among people who worked hard but also made a sport [16-18].

Realized in our country analysis of the relation between ischemic heart disease factors and education level or workplace in researches POL-MONICA in Warsaw showed, that in Polish population and also in western countries is observed the tendency of decreasing the thread of hart diseases but mostly among people with higher education and higher property qualification [17]. Therefore physical workers become a group at increased risk of circulatory system diseases because of cumulation of many unfavourably influencing factors like: hard physical work, static effort, lack of extraprofessional physical activity, slight and quickly decreasing physical efficiency. In this professional group are there are more people smoking, drinking and eating irrationally [17-19].

Despite of many investigations there is no answer on the question, if hard physical work in occupational work can be taken similarly as a extraprofessional physical activity, as a independent factor reducing risk of heart disease. In the cross-sectional study it is indicated positive influence of physical activity in the occupational work on risk factors of ischemic heart disease [20]. It was also indicated that this kind of activity does not prevent and does not increase the risk of cardiac infarct, however, it increase the risk of general mortality.

It is indicated in our research, that in tested group of employees doing hard and very hard manual work in comparison with employees performing light and moderate manual work, spondylopathy and some of circulatory system diseases like ischemic heart disease or disturbances of the cerebral circulatory occurred statistically more frequent. Disturbances of the cerebral circulatory, spondylopathy had also higher morbidity rates in the group of working hard and very hard in comparison with factory management workers, but differences were statistically non significant.

Conclusions

1. For employees performing hard and very hard physical work the morbidity rate connected with peripheral nervous system diseases was higher in comparison with the morbidity rate for mental workers.
2. In group of employees doing hard and very hard manual work the spondylopathy and some of circulatory system diseases like ischemic heart disease or disturbances of the cerebral circulatory occurred statistically more frequent than at employees performing light and moderate manual work.

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5-aminolevulinic acid 184
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In the report, Borowska M., Oczeretko E., Mazurek A., Kitlas A., Kuć P.. Application of the Lempel-Ziv complexity measure to the analysis of biosignals and medical images. Annales Academiae Medicae Bialostocensis 2005; 50 (Suppl. 2), 29-30; the results should have read:

Results

The mean values of Lempel-Ziv complexity for biomedical signals are: L-Z(s1)=0.481±0.060; L-Z(s2)=0.769±0.111, and L-Z(s3)=0.165±0.074. The high L-Z complexity value for respiratory rate signal indicates that this time series is close to unstructured randomness.

The mean values of Lempel-Ziv complexity for angiogenic patterns are: L-Z(A)= 0.0501±0.0199; L-Z(B)=0.0673±0.0263, and L-Z(C)=0.0854±0.0246 (p=0.0014, not-balanced ANOVA). The Lempel-Ziv complexity values were growing with the FIGO stage of disease.

The publisher wishes to apologise for this error.

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3. DeVita VTJ, Hellman S, Rosenberg SA. Cancer: Principles and Practice of Oncology. 4th ed. Philadelphia: J.B. Lippincott Co.; 1993.
4. Norman IJ, Redfern SJ, editors. Mental health care for elderly people. New York: Churchill Livingstone; 1996
5. Phillips SJ, Whisnant JR. Hypertension and stroke. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis, and management. 2nd ed. New York: Raven Press; 1995, p. 465-78.

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