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Cover Illustration: Schema of pathogenesis of inflammatory bowel disease and potential therapeutic targets. The different steps from antigens/bacteria crossing the epithelial barrier to phagocytosis and antigen presentation by antigen-presenting cells followed by T-cell activation and subsequent release of pro- and anti-inflammatory mediators and the migration of inflammatory cells via adhesion molecules is shown.
(Siegmund B, Zietz M. Roczniki Akademii Medycznej w Białysmstoku, 2004; 49, 22-30)

Surgical challenges in the twenty-first century

Ihse I

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To know where we are going, it is important to understand where we have been. Remarkable advances took place in health care delivery during the last part of the twentieth century profoundly changing the practice of surgery. Such advances include the development of imaging techniques, minimal access operations, endoscopy, catheter-based therapies, laser, information technology, and computer-based surgery. The coalescence of these innovations with unique achievements in molecular biology, molecular genetics, and pharmacogenetics will pave the way for further improvement of the standard of surgical care and stimulate future surgeon-scientists to keep surgery at the pole position. The old days were good, but they are gone. Now we must rise to meet numerous challenges ahead as our world is developing not only in science and surgical technology but also in demography, health care structures, economy, communication and the public's overall knowledge, expectations and demands. Some of these challenges will be discussed in more detail in this paper.

Societal and systemic forces

The development of high-speed communications has made the world smaller and human migration and mobility are on the point of equalizing the global diagnosis panorama. The travel time from the most distant country is shorter than the incubation period of most infections [1]. Thus, the perspective of surgery should also be global.

Among the changes which will have a significant impact on surgery is aging. People over 85 is a rapidly growing group in the Western world and they are the most likely to have chronic care needs. As surgeons play and will continue to play an important role in the management of cardiovascular disease, cancer, diabetes, and joint-and neurodegenerative diseases we are encouraged to elaborate a strategic approach for these growing patient groups.

In Sweden like in many other Western countries there are since some ten years more women than men entering medical school. Still, however, there is a substantial gender gap in the composition of surgical staffs. We must, thus, build flexibility into residency work schedules so that women can incorporate pregnancy and motherhood into their years of surgical training. At the same time as there are proportionally more women in the medical school the overall number of applicants are decreasing as are those choosing surgery for residency. This is to some extent explained by the changes in lifestyle now openly adopted by young surgeons, irrespective of gender. They want shorter workweeks and more time for their families and other values in life. Here we have an urgent challenge to make any effort to attract the best young people back to surgery.

The advent of the internet, teleconferencing, and e-mail has dramatically changed the speed and quality of worldwide communications. Patients are now ready to present us with the "latest and greatest" information. The well-informed public will have profound effect on the practice of surgery and patients will play a more dominant role in their own care. They will choose those hospitals and doctors who in an evidence-based way show the best and safest outcome of their treatment programs.

Surgical education and training

The knowledge explosion in medicine is a principal cause of development of the specialization seen during the last two decades. The rapid and profound advances in medical technology has increased the complexity of surgical, interventional and intensive care and fueled further specialization and subspecialization. In countries like Sweden the limited workhours

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(40 hours a week) in addition have enforced subspecialization so that the surgeon get a reasonable volume of patients within his or her specific area of interest [2]. Thus, the general surgeon in the true sense of the word, is an endangered species in our country as in most other Western countries. Young surgeons start early focused training in upper abdominal, colorectal, endocrine, breast, or vascular surgery; in the university hospitals, upper abdominal surgery is often divided into hepatic/biliary/pancreatic surgery versus upper gastrointestinal surgery. Subspecialization is probably a major cause of the improved outcome after surgery, which we have achieved in recent years. It has, however, negatively influenced on call work because modern Swedish surgeons do not always possess sufficiently broad surgical experience. Furthermore, the newly hatched specialists (normally about five years resident training program) are not ready to take independent responsibility for hospitalized patients in general surgical wards. A training period as junior specialist of six to eight years is normally required before they gain promotion as consultants. This situation largely reflects the low resident caseload in Sweden. A challenge for the future is to find ways to secure both the quality of highly specialized surgical care and that of basal care and emergency surgery.

Interdisciplinary care

As a consequence of the knowledge explosion and specialization/subspecialization health care has shifted form being specialty-based to be disease-diagnosis-or problem – based meaning that patients will be expected to be cared for in an environment based on disease rather than the method of treatment. It is logical to envisage surgeons as leaders of teams of specialists including e.g. HPB-surgeons, oncologists, radiologists and pathologists for the management of patients with liver, pancreas, bile duct, and gallbladder cancer. Even if the skill of the individual surgeon is important it seems to be even more crucial that the multidisciplinary treatment teams develop substantial experience in the management of the patients. It is becoming more and more difficult for the different specialists to defend their turfs and a future challenge is for all of us to open our minds and start walking side by side for the sake of our patients. This will link us together for a shared process of diagnosis, treatment, care and research.

The volume-outcome relation

There is considerable evidence that patients undergoing various kinds of complex treatments or high-risk surgical procedures have lower mortality rates and otherwise better outcomes if care is provided in centers that have a high caseload of patients with the same condition than if care is provided by hospitals with low caseload of such patients. In 1977 we reported that senior surgeons especially trained in pancreatic surgery had significantly lower hospital mortality after total pancreatectomy than the general surgeon undertaking such operations once in a while [3]. Two years later the first study dealing exclusively with the volume-outcome association was published by Luft et al. [4]. Their seminal observations of a relation between higher volume and better outcome have been supported by approximately 300 reports in the English-language literature. Most of the studies have analysed the effect of volume on hospital mortality. Compilation of earlier studies suggested that hospital volume had a greater impact on the outcome than the volume of the individual surgeon [5]. Recently, however, Birkmeyer et al. in an extensive study on eight different surgical procedures convincingly showed that the observed association between hospital volume and operative mortality was largely mediated by surgeon volume, though, to a degree that varied according to the procedure [6]. Some of the studies have included information showing lower complication rate at high-volume hospitals after e.g. esophageal, pancreatic, prostatic and thyroid surgery. Other authors have reported shorter postoperative stay at high-volume centers. If complication rate is low and hospital stay short, the cost should be reduced as well. This has been documented for at least four cancer operations and recently also for bariatric surgery [7].

In addition to the influence of hospital volume on the early surgical outcome, there is an increasing bunch of evidence suggesting that patients live longer after operations at high-volume centers for cancer of e.g. the rectum, colon, pancreas, lung and breast [8].

The majority of studies on the topic are done in the USA [9]. Recent reports from Canada, UK, The Netherlands and Finland have, however, come to the same conclusions as the US ones [10-13]. Still the referral pattern has remained practically unchanged in most countries, and few if any signs of regionalization of complex procedures have been seen. In the Netherlands, 40% to 46% of pancreaticoduodenectomies continue to be done in low volume units [12] and such operations are practiced in 50% of Swedish hospital, most of which do less than three operations annually [14]. However, with more and more patients seeking information on the outcome of surgical treatment the reality is that regionalization will get going and will continue to evolve.

Advances is science and technology

As mentioned above profound advances in science and technology during the last two to three decades is already changing and improving surgical practice. Technological innovations have previously had a major impact on the progress of clinical practice of orthopedics, neurosurgery, ophthalmology, otolaryngology, and urology, and more recently cardiac and vascular surgery. Advances in imaging, faster computers, and advanced software will influence the way we offer clinical solution to the patient's problem. The other side of the coin is the need for evaluation of the technology and its application, its introduction into practice, and the training of surgeons. For training medical simulators will be increasingly required and they will also have the capacity to assess the technical competence of the surgeons, which certainly will be a challenge to most of us as it was to pilots when similar measures were taken by the aviation industry a couple of decades ago.

Increasing demands on risk-free operations

After the report, To err is human, was published by the Institute of Medicine in USA in 2001 there has been an increasing focus in the Western world on how to improve patient safety [15]. These efforts take place at the same time as diagnostic and therapeutic procedures due to the paramount advances in technology are becoming more and more complex and intricate putting increased demands on the competence, skill and judgement of those who deliver the care. We take as surgeons minor or major calculated risks almost every day e.g. when we operate on ruptured aortic aneurysm, myomatous uteri, gallbladders or on tumours in frail and elderly patients weighing the risks of the disease against the risks of the procedure. The increasing use of prophylactic operations for genetically predestined malignant disease is a new challenge to us that will request practically risk-free operations as we in fact are operating on healthy individuals. Will parents accept any risk either as hypoparathyroidism or recurrent nerve injury in a child less than five years with the RET proto-oncogene identified? This question was asked by Murray Brennan, who coined the word preemptive surgery to this kind of prophylactic operations [16].

The overwhelming challenges for the twenty-first century is to develop surgery in such a way that the patients will be managed with optimal safety and optimal outcome. I have discussed some areas in this paper which we need to tackle to be able to approach these goals. The ideas I have suggested are just presented as food for thought.

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A comparative morphological study of direct nerve implantation and neuromuscular pedicle methods in cross reinnervation of the rat skeletal muscle

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Abstract

Purpose: The study was undertaken to evaluate the recovery of rat skeletal muscles reinnervated by crossed direct nerve implantation and crossed neuromuscular pedicle graft.

Material and methods: The animals (157) were divided into 3 groups. In the first group – direct nerve implantation – (DNI) 52 animals, the peroneal nerve was transected and implanted into the gastrocnemius muscle of the hind limb of the rats. In the second group (53 animals) – the neuromuscular pedicle (NMP) of the peroneal nerve was elevated and transferred to the gastrocnemius muscle. The third group consisted of 52 healthy animals. Muscle function was examined by electrophysiological methods (evoked electromyography and muscle isometric tetanic tension) and by morphological methods (reinnervated muscle weight, microscopic examinations, morphometric and histochemical examinations).

Results: The weight of reinnervated muscles in the first 3 weeks decreased. The lowest values of muscle weights were noted at 4 weeks. At the end of the experiment muscle weight in the first group was 64.3% of the control group and in the second group 65.2%. Morphometric, histological and histochemical analysis were performed after 12 and 36 weeks. At 12 weeks of the experiment the diameter of reinnervated muscle fibers in the second group was statistically higher than in the first group. At that time the process of reinnervation was more advanced than in the first group. At 36 weeks there were no statistical differences between the two groups. An increase in the number of muscle fibers was noted as the processes of reinnervations progressed.

At the site of nerve (or pedicled nerve-muscle) implantation new motor end plates were formed.

Conclusions: We consider that reinnervation of the experimentally paralyzed muscle is possible after crossed DNI and after NMP neurotization. The reinnervation with the NMP technique is quicker than with the DNI. Based on the morphological examinations – both methods guarantee only a partial recovery of the function of the paralyzed muscle.

Key words: denervation, direct neurotization, neuromuscular pedicle, muscle reinnervation, motor end plates.

Introduction

When the nerve is injured near its entrance to the muscle belly we cannot perform conventional methods of nerve repair (nerve suture, or nerve grafting) because we do not have the distal stump of the injured nerve. One useful method in such a situation is direct implantation of nerve fibers into the paralyzed muscle (direct nerve implantation method – DNI) [1-15]. We call such procedures direct neurotization of the muscle. The first experimental investigations of direct nerve implantations into paralyzed muscles were performed by Heinecke and Erlacher [16,17]. Steindler, Aitkin, Hoffman and Miller also contributed to investigating the processes of nerve regenerations. All these investigations showed that an injured proximal nerve stump can grow into the paralyzed muscle and establish functional connections (motor end plates). Direct nerve neurotization such as the nerve grafting procedures are inevitably connected with processes of retrograde Wallerian degeneration of the injured nerve [18,19]. Such problems can be avoided when we transpose intact undamaged nerve fibers

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to target organ [20,21]. In most human muscles the end plates are localized at places of nerve entrances into the muscle belly. This enables excision of the area of muscle containing the motor end plates with its motor nerve and transferring such a „nerve muscle pedicle" onto another paralyzed muscle. The concept of motor nerve transposition (MNT) was first reported in 1970 by Tucker et al. [1,22-29]. These authors called such a procedure "neuromuscular pedicle reinnervation" (NMP). This technique was successfully used for reinnervation of the human larynx [25-27]. Experimental studies of muscle reinnervation with motor nerve transplantation technique showed conflicting results. Some authors wrote about evidenced signs of motor reinnervation [1,24,30] other authors [31,32] doubted whether this method can really give successful reinnervation of paralyzed muscles. Most of the experiments made previously were performed on small paralyzed muscles of the larynx or face [33-35]. Only demonstration of satisfactory paralyzed extremity muscle reinnervation can confirm the technique of motor nerve transplantation to be another reliable technique of muscle reinnervation.

We performed our study to answer the following questions:

1. Is it possible to reinnervate a paralyzed hind limb rat muscles with the method of „motor nerve transplantation"?
2. What is the efficacy of reinnervation of a paralyzed muscle with the technique of neuromuscular pedicle (NMP) as compared with the method of direct nerve implantation (DNI)?
3. What is the extent of reinnervation of paralyzed muscles as evaluated by morphological methods?

Materials and methods

157 Wistar rats with an average weight of 250 gm were used. Anesthesia was induced with an intra-peritoneal injection of Thiopental at the dosage of 40 mg/kg of body weight. A curved incision was made on the posterior surface of the right thigh, a posteriori-based skin flap raised and biceps femoris muscle was pushed aside to expose the popliteal fossa. The tibial, common peroneal and sural nerves were identified. A 1.5 cm segment of tibial nerve was resected where it entered the gastrocnemius muscle and the proximal stump of the tibial nerve was implanted into the underlying adductor muscle. The whole sural nerve was also resected and the nerve implanted into the adductor muscle. The animals were operated on using microinstruments (Aesculap) and an operating microscope OPM-1.

The rats were divided into the three groups for the subsequent experimental procedures.

Group I (DNI) (52 animals). The common peroneal nerve was transected at the place where it traversed the fibular head. The proximal stump was transposed into the proximal third of the simultaneously denervated gastrocnemius muscle (cross neurotization). The epimysium of gastrocnemius muscle was incised and the peroneal nerve stump was implanted into the bluntly created muscular pocket (*Fig. 1*, *2*). If there was bleeding when creating a pocket another muscular pocket was prepared. A single 10/0 nylon suture, transfixing the epimysium and epineurium of the peroneal nerve stump was used to stabilize the nerve. The biceps femoris was replaced and the wound closed.

Group II (NMP) (53 animals). The peroneal nerve of the right leg was exposed around the fibular head and further into the muscle belly. A small piece of tibial anterior muscle (0.2/0.2 cm) – containing intact motor endplates with the entering peroneal nerve fibers was sharply excised (nerve muscle pedicle) and transposed to the proximal third of the simultaneously denervated gastrocnemius muscle (*Fig. 3*, *4*). At that site, where the small block of pedicled muscle was approximated – the epimysium from the gastrocnemius was removed. Two stay nylon sutures 8/0 between the peroneal nerve muscle island and epimysium of the denervated gastrocnemius muscle were used to stabilized the muscle. The wound was closed in layers.

Group III (52 animals). The group of healthy non-operated rats served as the control.

Morphological, histological and enzyme histochemical assessment

After electrophysiological and functional assessment (36) the GAST-PL-SOL muscle group was excised en bloc, the macroscopic appearance of neurotized muscles was evaluated and, the wet weight of the muscles estimated. With the use of a computer program "Imager-512" morphometric measurements (a mean muscle fiber count per 1 mm^2 area and size of muscle fibers) of reinnervated muscles was performed.

Small muscle blocks from the reinnervated gastrocnemius with the pedicle were fixed in buffered 10% formalin solution and than embedded in parafin blocks. Microscopic examinations of cross-sections of the reinnervated muscles stained by the hematoxylin-eosin method were performed.

Muscle blocks collected with a pedicle (common peroneal muscle or pedicled muscle island) were immersed in liquid nitrogen, cut in a cryostat into pieces of 10 μm thickness and stained for demonstration of acetylcholinesterase activity by the method of Tsuji [37]. By this method it was possible to demonstrate motor end plates beneath the nerve pedicle graft.

The results obtained were statistically assessed. Differences between mean values were analyzed using Student's t-test (data of normal distribution) or the Mann-Whitney U test (data without normal distribution). The differences were recognized as statistically significant when $p<0.05$. Statistical analysis was performed with a computer using Statistica program.

Results

During the experiment 157 rats were operated on with the two methods mentioned above. Five rats died in the perioperative period (probably because of overdosing of the anesthetic drug), 2 rats died during the later periods of the experiment. 150 animals were examined and assessed.

In the first weeks of the experimental study, the weights of the reinnervated muscles in the DNI and NMP groups decreased (*Tab. 1*). In the second week, the muscle weight in the DNI group was 1490 (±65) mg, and in the second group 1798 (±75) mg – 75.7% and 91.3% of the controls respectively.

Figure 1. The proximal stump of the common peroneal nerve was transposed and implanted into the simultaneously denervated gastrocnemius muscle

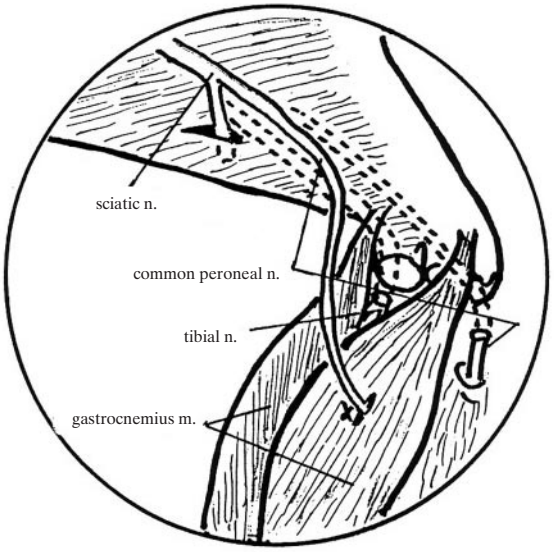


Figure 3. The neuromuscular pedicle was excised, transposed and implanted into the denervated gastrocnemius muscle

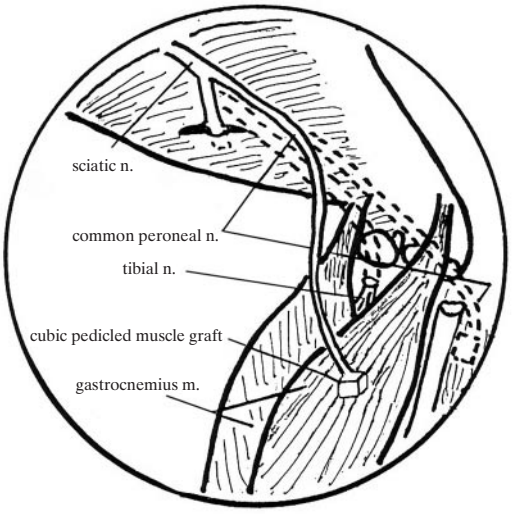


Figure 2. Intraoperative view – the common peroneal nerve is inserted into the pocket of the gastrocnemius muscle

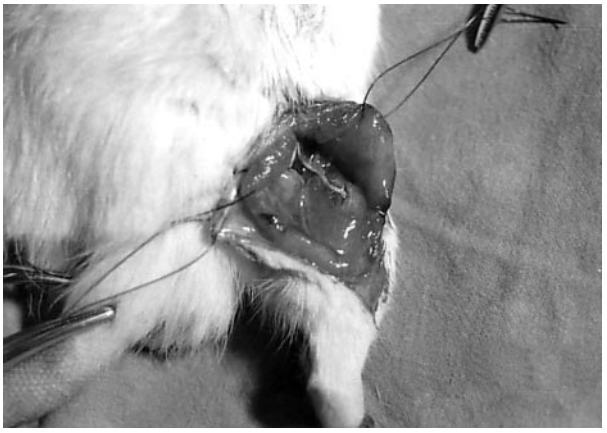


Figure 4. Intraoperative view – the prepared pedicled muscle pad is inserted into the gastrocnemius muscle

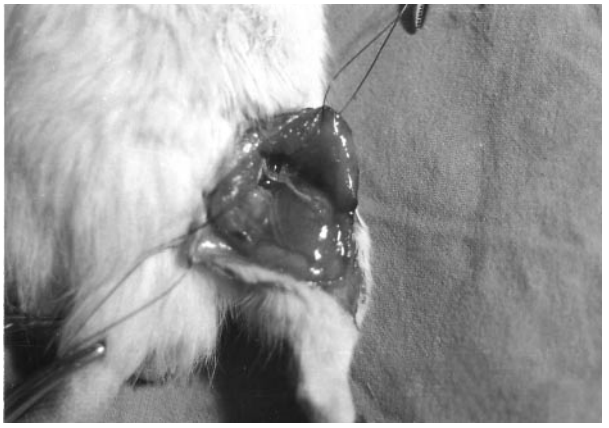


Table 1. Mean data of reinnervated GASTR-PL-SOL muscle weights. In the lower right-handcorner the number of rats from which the mean value was calculated is shown. # Data estimated before experimental study. * Statistically significant difference in comparison with the NMP and controls ($p<0.01$). **Statistically significant difference in comparison with the controls ($p<0.05$). *** Statistically significant difference in comparison with the DNI and controls ($p<0.01$)

Time after surgery (weeks)	Group I Direct nerve implamation	Group II Neuromuscular pedicle	Control group
	Muscle weight (mg)	Muscle weight (mg)	Muscle weight (mg)
0#	1954±77 N=20	1950±62 N=20	1951±42 N=10
2	1490±65 * N=8	1798±75 *** N=8	1969±118 N=8
4	1014±118 * N=8	1531±76 *** N=7	2217±164 N=8
12	1245±117 * N=7	1634±118 *** N=8	2674±213 N=8
16	1332±69 * N=8	1674±162 *** N=8	2819±190 N=8
24	1752±178 ** N=8	1825±249 ** N=8	2993±190 N=8
36	2135±195 ** N=8	2165±123 ** N=8	3321±374 N=7

Figure 5. **Appearance of muscle after 36 weeks of experimental study**

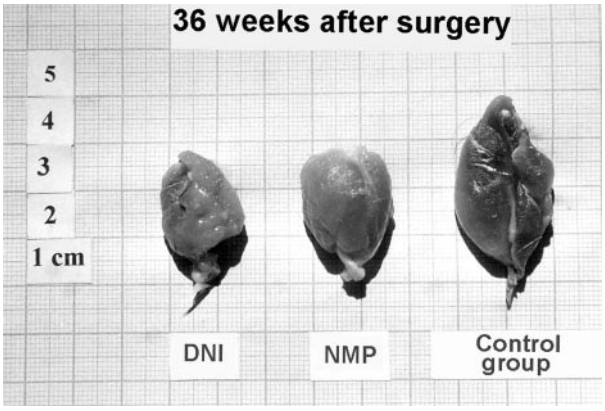


Figure 6. **Computer muscle weight curves of experimental groups**

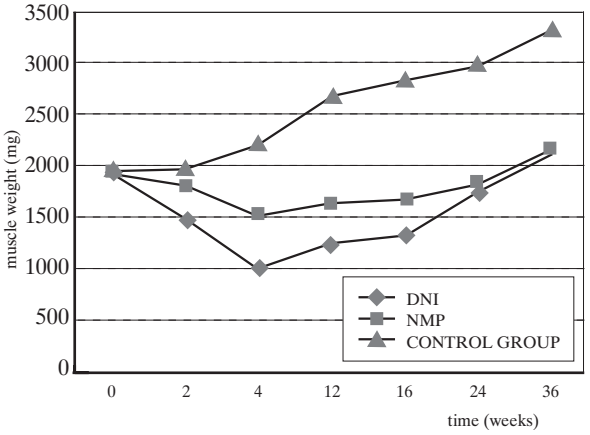


Table 2. **Muscle fiber counts and diameter in the experimental groups. In the lower right-hand corner the number of animals from which the mean value was estimated is shown. * Significant differences in comparison with the DNI and control group (p<0.01). **Significant differences in comparison with the NMP and the control group (p<0.01)**

Time after surgery (weeks)	Group I Direct nerve implantation DNI		Group II Neuromuscular pedicle NMP		Control group	
	Muscle fiber counts (mm^2)	Muscle fiber diamater (μm)	Muscle fiber counts (mm^2)	Muscle fiber diamater (μm)	Muscle fiber counts (mm^2)	Muscle fiber diamater (μm)
12	605±65.2* N=6	12.1±4.11* N=6	721±78.1** N=6	18.5±4.85 N=6	998±78.3 N=8	24.6±1.8 N=8
36	882±67.5 N=7	22.8±4.55 N=7	901±69.4 N=7	25.9±7.21 N=7		

At that time the mean muscle weight of the DNI group was significantly lower than in the NMP (p< 0.01) and control group (p< 0.05). There were also significant differences between the NMP and control group (p<0.05). After 4 weeks, the muscle weight in both groups reached its lowest value (DNI group 45.7% of the normal group, and NMP group 69.1% of the normal group). There were statistical differences between the DNI and NMP groups at that time (p<0.01).

From the 4th week onwards the muscle weight in two groups successfully increased (*Fig. 6*). Between the 12th week and the end of the experimental study the increase in muscle weight was less dynamic. In the 16th week the muscle weight in the DNI group was 1332 (±69) mg and in the NMP group 1674 (±162) mg. The differences between the DNI and NMP groups (p<0.01), DNI and control (p<0.01), and NMP and control (p<0.01) were found to be significant. At the end of the experimental study (36 weeks) the muscle weight of the DNI group was 64.3% and NMP group 65.2% of the control rats (*Fig. 5*, *6*). No statistical differences between DNI and NMP groups were noted, although the muscle mass from the DNI group was significantly lower than in the control (p<0.01), and similarly the NMP muscle weight was significantly lower than in the control group (p<0.01).

Morphometric analysis was performed after 3 and 6 months (*Tab. 2*). The number of muscle fibers present in a cross-section of 1 mm^2 reinnervated muscle taken from the site of nerve (island) implantation and the diameter of the reinnervated muscle fibers were estimated. After 12 weeks the mean fiber count on the cross-sections in the DNI group was 605/mm^2, and in the NMP group 721/mm^2. At that time the number of muscle fibers in the control group was 998/mm^2 (*Fig. 7*). There were significant differences between the DNI and NMP groups (p<0.01), DNI and control rats (p<0.01) and NMP and controls (p<0.01). After 36 weeks the number of muscle fibers was greater in the NMP than in the DNI group, although these values did not change significantly. The number of muscle fibers in the control group was greater than in the DNI and NMP groups, no significant differences between the controls and DNI or control and NMP groups were noted.

After 12 weeks the muscle fiber diameter in the DNI group was 12.1 μm and in the NMP group 18.5 μm. The mean fiber diameter in the control rats was 24.6 μm. There were significant differences between the DNI and NMP groups (p< 0.01), and the DNI and control groups (p< 0.01). After 36 weeks the mean fiber diameter in the DNI was smaller than in the NMP group (*Fig. 8*). These values did not change significantly (p< 0.01). No

Figure 7. **Fiber counts of reinnervated muscle in the groups examinated. * Significant differences in comparison with the NMP and control group (p<0.01). Significant differences in comparison with the DNI and controls (p<0.01)**

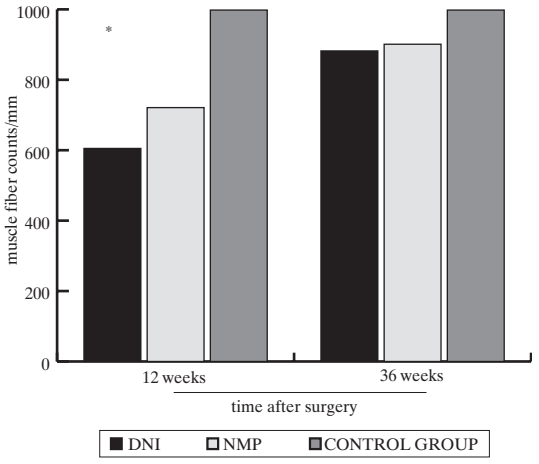


Figure 8. **Computer muscle fiber diameter of experimental groups. * Significant differences in comparison with the NMP and control group (p<0.01)**

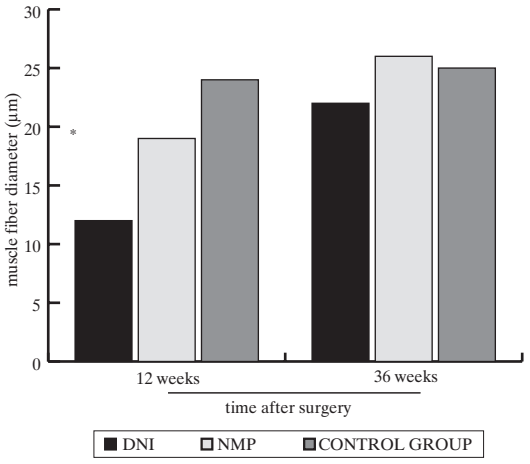


Figure 9. **Histological pattern in the DNI group (most muscle fibers are placed in regular fascicules, the diameter of the fibers are similar, Transvers sections of fibers appear to be oval and polygonal, site of nerve implantation, DNI method 36 weeks after surgery, H+E stain, x200)**

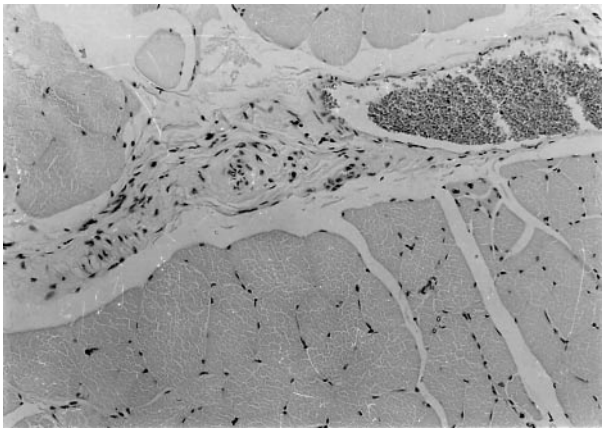
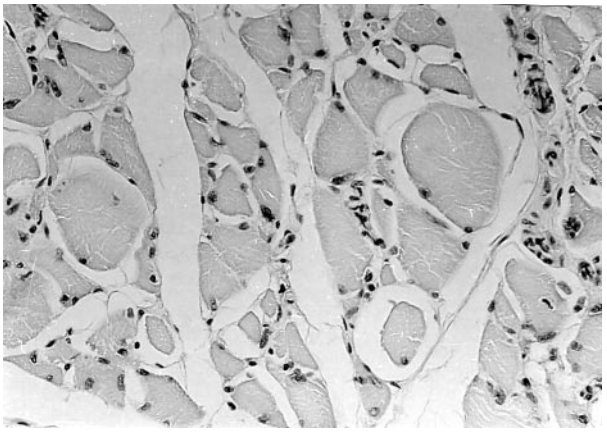


Figure 10. **Histological pattern in the NMP group (most fibers are large, hypertrophied, another fibers are polygonal atrophied, site of nerve-muscle pedicle aproximation, NMP method, 36 weeks after surgery, H+E stain, x200)**



significant differences were found between the DNI and control groups, and the NMP and control groups at that time.

Histological studies

In the 4th week muscle fibers of different shape and diameter were seen in the DNI group. Some of them were rounded, others polygonal shaped. Small branches of nerves were evident on the cross-sections of the reinnervated muscle. In the later periods of reinnervation (12th week) most of the round or polygonal-shaped muscle fibers with similar diameters gathered in regular fascicules (*Fig. 9*).

In the NMP group, in the late periods of reinnervation, fascicules of various shapes were seen (*Fig. 10*). Some fibers were large, oval shaped, while others were polygonal with signs of degeneration.

Histochemical examination

In NMP group after 4 weeks, motor end plates were demonstrated at the place of muscle island approximation. No acetylcholinesterase staining was seen in the DNI group at that time. After 6 weeks motor end plates of various shapes, arranged in irregular lines, sometimes incompletely stained, were observed in both groups. At the end of the experimental study, the completely-stained clusters of motor end plates were more rounded in the DNI as well as in the NMP group (*Fig. 11*, *12*).

Figure 11. **Motor end plates evident in the DNI group (irregular and linear forms of well stained motor end plates, 36 weeks, AChE method by Tsuji, x400)**

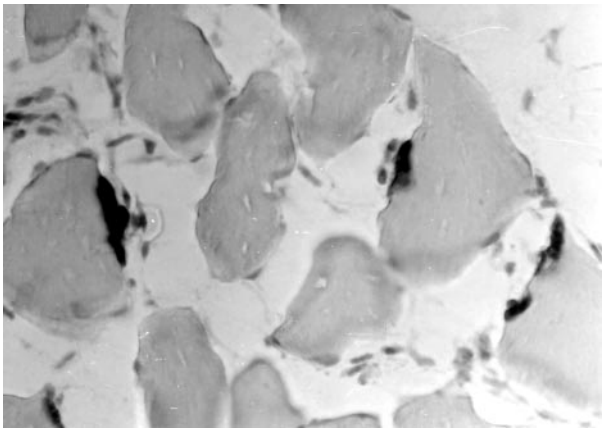
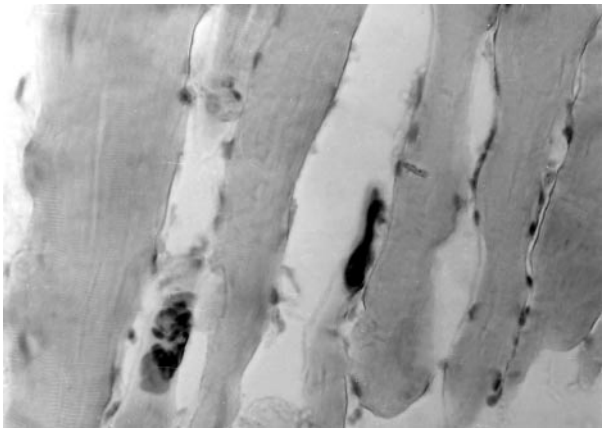


Figure 12. **Motor end plates evident in the NMP group, 36 weeks (oval and linear shaped motor end plates, well stained in histochemical examination, AChE activity – Tsuji method, x400)**



Discussion

The direct nerve implantation method (DNI) was popularized in Europe by Giorgio Brunelli [2-5,38,39]. The author divided the proximal stump of the injured nerves into fascicules to implant them into denervated muscle bellies. The neuromuscular pedicle – NMP technique was introduced by Tucker H in otolaryngological practice at the beginning of 1970 [25-29,37]. Tucker postulated that simple healing of the pedicle muscle into the denervated muscle belly is the primary prerequisite for transferring evoked active potentials from the island to the denervated muscle. What are the differences between these two techniques?

The DNI method is connected with retrograde Wallerian degeneration of transected nerve fibers. During excision of the pedicle muscle graft (NMP technique) there is complete transection injury to the terminal nerve branches at the cut margins of the muscle pad, where axons travelling to more distant muscle fibers are divided. The muscle island contains however a large amount of undivided axons with functional motor plates. The NMP technique enables the transferring of motor fibers with these functional end plates [20]. The retrograde Wallerian degeneration in such nontransected fibers is avoided.

In our experimental study, we neurotized the gastrocnemius muscle with common peroneal nerve (nerve stump or pedicle muscle). That means there was a crossed neurotization of the simultaneously paralyzed muscle. The reinnervation of a muscle with a "foreign" nerves always gave inferior results to reinnervations with native nerves [40]. Reports in the literature vary as regards the period of time from implantation of a "foreign" nerve to the first functional signs of its reinnervation that is from 2 to 12 weeks. The reinnervation was always more effective (in laboratory study and in clinical practice – when the proximal nerve stump was divided into fascicules). Resection of the epineurium surrounding the nerve stump reduces the possibility of connective tissue barrier formation, that can prevent nerve regeneration [41,42]. Brunelli and other authors divide the proximal nerve stump into as many fascicules as possible and implant them into various depths of the muscle belly to increase the counts of reinnervated muscle fibers [5,29,38,43,44].

In our experiment the proximal stump of the common peroneal nerve (CPN) was not divided into fascicles, which resulted in a decrease in the number of reinnervated muscle fibers. On the other hand, the three-dimensional pedicle muscle in the NMP technique contains the transected axons, which are considerably separated from each other. The NMP is similar to the originally described nerve implantation techniques dividing the proximal stump of an injured nerve into fascicules.

The crucial question connected with pedicle muscle technique is its blood supply. Can the muscle and nerve cells survive the transposition? In our experiment the CPN was separated with the alveolar tissue (sleeve), an effort was made to preserve as many small blood vessels in the pedicle as possible. We treated the muscle island as vascularized on a nerve and elevated with the nerve alveolar tissue. We did not determine how many muscle fibers survived such a procedure. In previous studies a special and considerable regenerative potential of autogenous muscle transplants was noted [45-48]. This also applies to the muscle island in the NMP method. A piece of muscle in the NMP method would be a source of growth of new muscle tissue and a considerable regeneration will occur in a muscle graft [45,46,49].

Pedicle muscle fibers can be a source of fibronectin and laminin, which create an excellent medium for the growth of regenerating nerve sprouts [20].

The structural basis for neuromuscular conduction properties are motor end plates. In our experiment, the "fast" nerve (CPN) reinnervated the paralyzed gastrocnemius muscle. The gastrocnemius muscle in contrast to the soleus muscle has no strict asymmetrically located native zone plate [50]. End plates in the gastrocnemius muscles are distributed widely throughout the muscle belly. According to Brooke [51] the gastrocnemius

muscle consists of type B fascicules (there are fewer type A and IIA fascicles than in the soleus muscle). A similar distribution of fascicules is found in the anterior compartment of the hind limb. The transposed neuromuscular pad contained similar fibers to those in the gastrocnemius muscle. We did not perform typical ATP-se staining. No conversion of muscle fascicles was anticipated.

Because there is no strictly well-located clear native zone plate in the gastrocnemius muscle – an increase in accumulated end plates around the implantation site of a nerve was the sign of reinnervation. At the 4th week after nerve (nerve-muscle) implantation, the motor end plates were seen in cross-sections in both groups. In the DNI group the plates were seen in those animals, in which electrophysiological responses were noted. Their elongated shapes, diffuse granular form little resembled a typically found "en grape" form. That's why we treat those plates as new (ectopic) motor end plates. At that time in the NMP group motor end plates resembling those of the DNI group, were seen, but also "en grape" forms that could be "old" or transposed with the muscle pad functional motor end plate. Without electron microscopy it is not possible to define the actual stem of such neuromuscular junctions. In the late periods of observations (36th week) there was not so great a diversity in the form and size of the motor end plates, the plates of both groups had a rounder disc-like appearance, they were clustered together. It was obvious that in the early stages of reinnervation there were more motor end plates in the NMP operated group. We know that new regenerating motor axons preferably reinnervate original motor end plates, which are located on only 0.1% of the surface of a muscle fiber [52]. On the basis of our study, we have found that regenerating fibers in two of the above – mentioned methods innervated old end plates, in the NMP method of reinnervation consideration must be given to functional motor end plates that were transferred with a pedicle neuromuscular graft.

On the basis of our experimental study we conclude that:

1. Reinnervation is possible by means of two methods of neurolization, DNI and NMP methods.
2. Reinnervation of muscles with the crossed DNI and NMP method only partially restores the morphology of the paralyzed muscles.

Acknowledgment

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Vasopressin and oxytocin in normal reproduction and in the patophysiology of preterm labour and primary dysmenorrhoea. Development of receptor antagonists for therapeutic use in these conditions

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Abstract

Vasopressin and oxytocin are synthesised in the hypothalamus and released to the blood stream via the posterior lobe of the hypophysis. Research during later years has shown that these peptides are also produced in other parts of the brain. The secretion to plasma is stimulated by oestrogen, an effect which is counteracted by progestagen. During delivery the fetus can also produce substantial amounts of vasopressin and oxytocin. Additionally, the uterus itself may be a source of these hormones and we have recently found oxytocin mRNA in the endometrium of non-pregnant women with the highest levels around the time of ovulation.

In the onset of labour preterm and at term pregnancy vasopressin and oxytocin are centrally involved and in primary dysmenorrhoea the former hormone seems to play a key role in the mechanisms of increased contractions and reduced blood flow in the uterus of the condition. In women with the latter condition the plasma concentration of vasopressin is several-fold higher than that in healthy control persons. Both in pregnant and non-pregnant women the myometrium is activated via specific vasopressin V_{1a} and oxytocin receptors. This vasopressin receptor is different from the vasopressin V_{1b} receptor of the anterior lobe of the hypophysis, which is important in mood changes and V_2 receptor of the kidneys mediating fluid reabsorption. At the onset of labour preterm and at term the vasopressin V_{1a} and oxytocin receptors are elevated to a moderate degree. In non-pregnant women the receptor density varies over the menstrual cycle and increase markedly at the onset of menstruation. Substances, which block the uterine vasopressin V_{1a} and oxytocin receptors inhibit preterm labour and primary dysmenorrhoea.

Key words: vasopressin, oxytocin, receptors, antagonists.

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Introduction

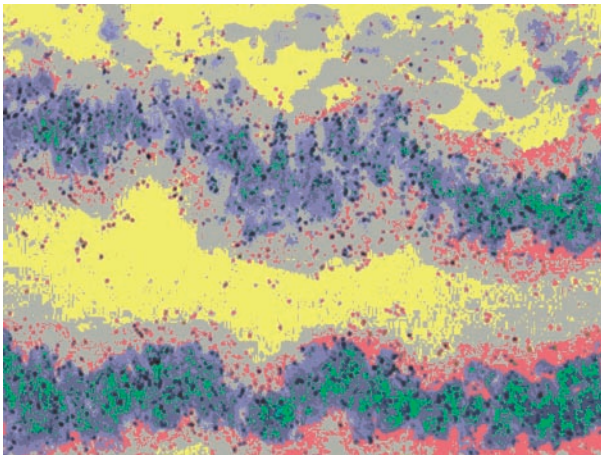
The importance of vasopressin and oxytocin as uterine stimulants has been studied extensively during recent years. Several important discoveries have been made and programs have started to develop antagonists to the uterine effect of these hormones. One of these antagonists, atosiban (Tractocile®, Ferring Pharmaceuticals, Denmark) has been registered for the treatment of preterm labour. This overview will briefly summarise this research.

Synthesis and secretion of vasopressin and oxytocin

Vasopressin and oxytocin are synthesised in the hypothalamus and released to the blood via the posterior lobe of the pituitary. Neurones containing these peptides have also been demonstrated in other parts of the brain than the hypothalamus [1]. The secretion to the blood stream of vasopressin and oxytocin in non-pregnant women is stimulated by oestrogen, an effect which is counteracted by progesterone [2-4]. The level of ovarian hormones also influences osmotically-induced release of vasopressin and oxytocin [5].

During the stress of labour the fetus produces vasopressin and oxytocin in substantial amounts [6]. Other fetal sources than the brain also probably exist, since circulating vasopressin and oxytocin have been demonstrated in anencephalic foetuses [7].

Figure 1. **Illustration of oxytocin mRNA detection (dots) by in situ hybridisation in endometrial gland of a non-pregnant woman at the time of ovulation**



Vasopressin and oxytocin are possibly also synthesised in the uterus itself. Immunoreactive vasopressin and oxytocin have been demonstrated in the non-pregnant human uterus, in particularly in cervical part of the isthmus [8]. These peptides have also been demonstrated in human follicular fluid, a finding indicating that the hormone is of importance for ovulation [9]. During human pregnancy immunoreactive vasopressin and oxytocin have been demonstrated in the myometrium [10]. There are also signs of formation of oxytocin in the placenta, fotal membranes and decidua in the human and the rat [11-13]. Recently, our group has demonstrated oxytocin mRNA in the endometrium of non-pregnant women by in situ hybridisation (*Fig. 1*) and real time PCR [14]. The amount of oxytocin in the endometrium seems to vary during the menstrual cycle, reaching the highest level around time of ovulation [14]. It is, however, unclear if oxytocin is released from uterine and foetal sites in amounts sufficiently high to have physiological and/or patophysiological effects.

Importance of vasopressin and oxytocin in activation of the uterus – effect of receptor-blocking substances

Pregnant uterus

Circulating vasopressin could play a role in the onset and regulation of human labour, but data regarding this is limited [15]. Oxytocin has since long been ascribed a major involvement in mechanisms of labour, both preterm and at term pregnancy. It is not established that the onset of labour is caused by increased plasma concentrations of oxytocin [16,17]. An importance of increased plasma concentration is supported by the finding that oxytocin is released in a pulsatile manner and that the frequency of pulses is increased as labour progresses [18]. A nocturnal peak in plasma concentration of oxytocin paralleling increased uterine activity has also been demonstrated [19]. However, women with diabetes insidipus usually have normal labour, even those who almost lack oxytocin [20].

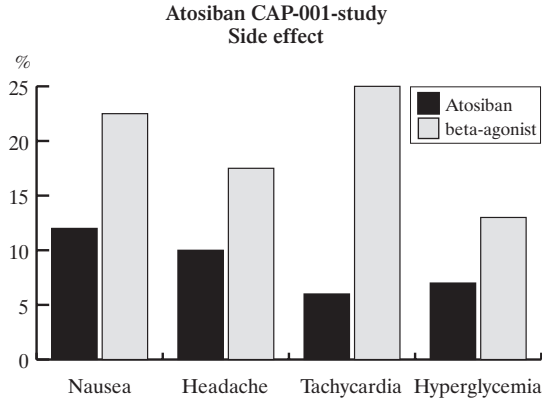
The pregnant uterus contains receptors for vasopressin and oxytocin [21,22]. Three different types of vasopressin receptors have been demonstrated, V_{1a} V_{1b} and V_2 receptors, whereas oxytocin only has one type of receptor. The vasopressin V_{1a} receptor is distinctly different from the receptor of V_2 type, which regulates kidney function and the V_{1b} receptor of the anterior pituitary. Results from studies of receptor binding indicate that vasopressin in the myometrium acts both over its own receptor, V_{1a}, and to some extent of the oxytocin receptor [23]. Oxytocin on the other hand acts mainly over the oxytocin receptor, but activates the myometrium also to some extent via the vasopressin V_{1a} receptor [23]. The effect of vasopressin in the pregnant uterus is somewhat more pronounced than that of oxytocin and the same relation exists also regarding the number of binding sites for the two peptides [22].

A markedly increased number of oxytocin receptors in the uterus was previously believed to be the mechanism initiating labour preterm and at term pregnancy [24]. More recent studies have only shown a tendency to an increase in protein and mRNA for the oxytocin receptor in connection with the onset of labour [21,22,25]. Neither are there any firm proofs for a substantial upregulation of the vasopressin V_{1a} receptor at the onset of labour [22,26]. However, individual myometrial cells can show great heterogenocity and rapid changes in their expression of oxytocin receptor at the onset of labour [27]. Spontaneous contractions in vitro of human myometrium anyhow seem to be dependent on the oxytocin receptor [28]. The difficulty to confirm an importance of increased plasma levels of vasopressin and oxytocin or elevated receptor density in the myometrium at the onset of labour suggests that also other mechanisms may be involved. A local synthesis in the uterus with paracrine effect could be such a mechanism.

The importance of vasopressin and oxytocin for the initiation of labour preterm is confirmed by the therapeutic effect in this condition of an antagonist to the oxytocin and vasopressin V_{1a} receptor, atosiban [29,30]. This substance was discovered and developed by author of this survey together with scientists at Ferring AB in Malmö, Sweden. Atosiban has been shown to be at least as effective in postponing delivery as the previously used treatment, β_2-adrenoceptor stimulating substances, but having far less side effects [31] (*Fig. 2*). The time when the uterus is relaxed can be used for treating the underlying cause of labour, which in many cases, e.g. urinary infection, can be eliminated [32].

The usefulness of blocking vasopressin V_{1a} and oxytocin receptors in preterm labour is confirmed by results from a newly completed study with the orally active antagonist SR 49059 [33]. A marked inhibition of labour was observed in patients receiving the compound, whereas contractions were more or less unchanged in a control group of women treated by placebo [33]. Comparative studies of the peptide substance atosiban and the non-peptide, steroid-shaped SR 49059 showed a similar receptor binding and inhibiting effect on isolated myometrium from pregnant women delivered preterm and at term pregnancy [23]. In non-pregnant volunteers a similar inhibitory effect on

Figure 2. **Side effects of atosiban and betamimetrics in a worldwide comparison involving 742 patients [31]. Three cases of pulmonary oedema also occurred at treatment by betamimetrics, but no such complication after atosiban**



vasopressin- and oxytocin-induced contractile activity was also observed [34,35].

Atosiban has a more pronounced antagonistic effect on the vasopressin V_{1a} than on the oxytocin receptor [36,37]. A new analogue has now been discovered, barusiban, which has an equal potency to that of atosiban in inhibiting oxytocin effects on myometrium from preterm and term pregnant women, but lacks effects on the vasopressin V_{1a} receptor [38,39]. This substance could be a valuable tool in elucidating the relative importance of vasopressin V_{1a} and oxytocin receptors for activating the human uterus preterm and at term pregnancy. Our group is also involved in testings in vitro and in vivo on healthy volunteers of new vasopressin V_{1a} and oxytocin antagonists, studies which could lead to important advances in the understanding of mechanisms activating the human uterus.

Non-pregnant uterus

Vasopressin is an important pathophysiological factor in the increased myometrial activity and reduced uterine blood flow of primary dysmenorrhoea [40]. Women with primary dysmenorrhoea have an increased plasma concentration of vasopressin [41-43]. Vasopressin activates the smooth muscle activity of myometrium and uterine arteries in non-pregnant women via vasopressin V_{1a} receptors and to some extent via oxytocin receptors [44]. The vasopressin V_{1a} receptor can possibly have two subfractions, one for activating in the myometrium and one for stimulating the smooth muscle of vessel walls [45]. Oxytocin is probably less important than vasopressin in non-pregnant condition, since the amount of oxytocin receptors in the myometrium and the effects of the peptide are both about five times lower than those of vasopressin [23,44]. A role of oxytocin also in non-pregnant condition is however supported by the finding of a high amount of oxytocin mRNA in endometrial glandular cells of non-pregnant women, particularly around the time of ovulation [14].

In studies of uterine arteries from non-pregnant women undergoing hysterectomy we found that the smallest arteries, the so called resistant vessels are the most sensitive ones [46,47]. Vasopressin is highly active on the vessels followed in order by endothelin, oxytocin and noradrenaline [46,47].

The etiological importance of vasopressin and oxytocin in primary dysmenorrhoea is confirmed by the therapeutic effect in this condition of substances, which block vasopressin V_{1a} and oxytocin receptors [48,49]. Both spontaneous and vasopressin-induced myometrial activity in dysmenorrheic women are inhibited by atosiban, in parallel to a decrease in experienced pain. We have also shown that an endoperoxin-2 agonist inhibits both spontaneous and vasopressin-induced contractile activity in women, an observation which could be of therapeutical importance [50].

Conclusions

Preterm labour is the most therapeutic importance of preneonatal mortality and morbidity. Vasopressin and oxytocin seem to play key roles in this condition, the hormones being synthesised in the hypothalamus, by the fetus and, at least regarding oxytocin, in the uterus itself. The hormones stimulate contractions via vasopressin V_{1a} and oxytocin receptors, which may be upregulated to some extent in connection with onset of contractions. Receptor blocking substances may stop preterm labour contractions and the invention of atosiban has been a milestone in the treatment. However, preterm labour has several aetiologies and specific therapies have to be designed.

Menstrual pain is a problem in up to 50% of all non-pregnant women who have not had any children and in 10% they have to be absent from school or work because of pronounced symptoms. Vasopressin secretion is markedly elevated in primary dysmenorrhoea and the uterus is extremely sensitive to this hormone, much more than to oxytocin. Vasopressin V_{1a} and oxytocin receptor blocking agents may have a therapeutic potential also in this condition.

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Therapeutic approaches in inflammatory bowel disease based on the immunopathogenesis

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Abstract

Our understanding of the etiology and pathogenesis of IBD has improved extensively over the past years. At the center of the pathogenesis seems to be an excessive pro-inflammatory immune reaction towards normal intestinal flora. The different factors involved in this concept will form the focus of this review. The initial phase of antigen processing and presentation can be influenced by either modulation of the intestinal flora via antibiotics or probiotics or by direct stimulation of macrophages through GM-CSF treatment. Antigen recognition and activation of T-cells can be down-regulated by immunosuppressives such as azathioprine, CsA or methotrexate thus building the basis for current treatment in IBD. The pro-inflammatory character of the immune reaction is defined by the predominance of certain T-cell subpopulations. By targeting cytokines the disbalance of these subpopulation should be reconstituted. Here we will focus first on preliminary clinical as well as experimental data for the pro-inflammatory mediators IL-12 and IL-18 as well as for the anti-inflammatory cytokine IL-10. Second, the clinical data for the TNFα antibody that has been proven to be efficacious in Crohn's disease and the associated risks will be discussed. Last, recent clinical and experimental data on targeting cell adhesion as well as intracellular signaling pathways will be presented. In summary, with regard to this review, treatments, which intervene as early as possible in the initiation of the pathological immune reaction and simultaneously have a favorable side-effect profile, must be the focus of future research.

Key words: inflammatory bowel disease, mucosa, therapy.

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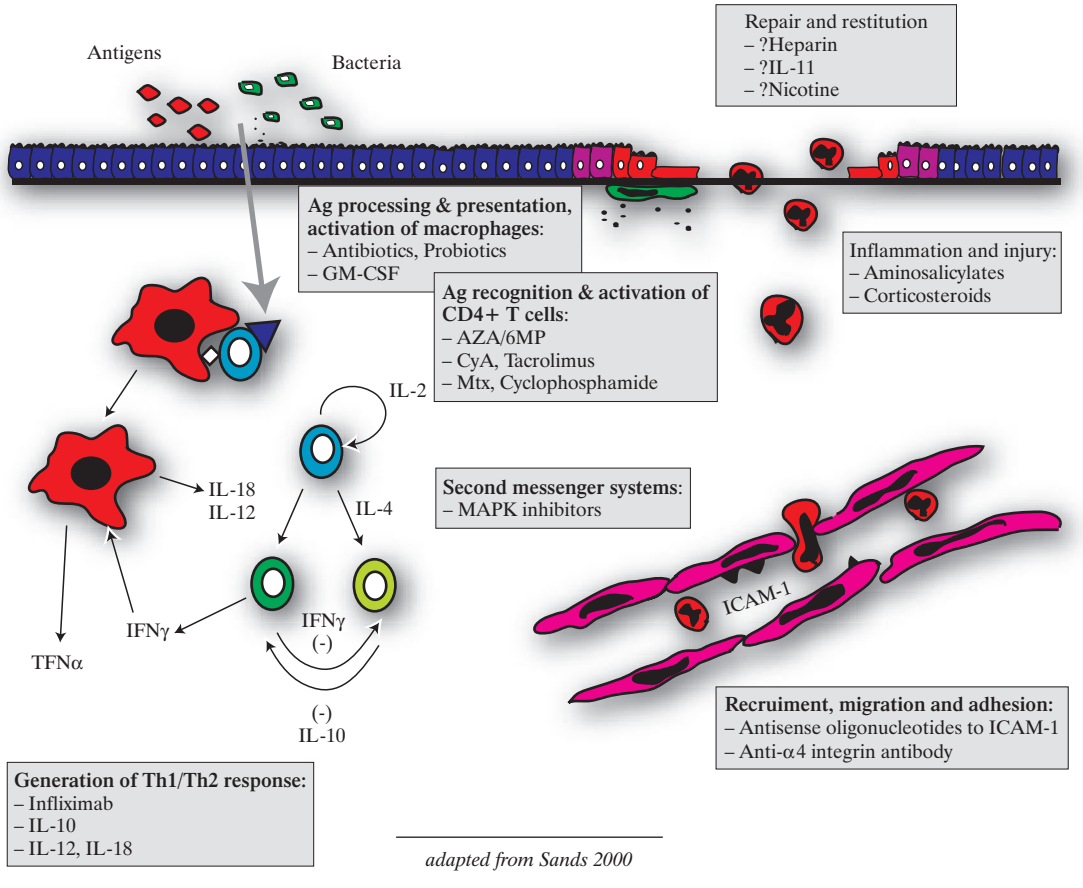
Introduction

The entire etiology and pathogenesis of inflammatory bowel diseases (IBD) is still unresolved, however, the understanding has improved extensively over the past years. In light of the diversity of substances and bacteria within the intestinal lumen, it is remarkable that the gut is not perpetually inflamed. The presence of low-level physiologic inflammation within the healthy intestinal mucosa represents a state of preparedness to deal with potentially harmful agents, but a more vigorous response would be not appropriate if directed toward the innocuous commensal flora of the gut. Inflammation is kept in check through an active process of immune tolerance. The dysregulation of the process of tolerance accompanied by an excessive pro-inflammatory immune reaction towards normal intestinal flora builds the current basis for the understanding of the pathogenesis of IBD, and furthermore, this concept creates the basis for distinct immunomodulatory approaches.

The present review will provide a systematical overview of the immunomodulatory approaches in IBD. As illustrated in *Fig. 1*, we will first focus on antigen processing, presentation and activation of macrophages and underline the therapeutic impact of probiotics and antibiotics. In addition, the potential role of granulocyte macrophage-colony stimulating factor (GM-CSF) therapy will be discussed. Activation of macrophages results in T-cell activation thus in a second step antigen recognition and activation of $CD4^+$ T-cells will build the focus. The unspecific suppression of the T-cell response by the classic immunosuppressive drugs azathioprine, cyclosporine A, methotrexate and cyclophosphamide and their efficacy in IBD

Figure 1. **Pathogenesis of inflammatory bowel disease and potential therapeutic targets. The different steps from antigens/bacteria crossing the epithelial barrier to phagocytosis and antigen presentation by antigen-presenting cells followed by T-cell activation and subsequent release of pro- and anti-inflammatory mediators and the migration of inflammatory cells via adhesion molecules is shown. The bold printed headings in the boxes indicate the different steps that will be discussed in this review**



therapy will be evaluated. In particular, the recently described mechanism of action for azathioprine will be summarized. The broad T-cell activation is followed by the generation of a T-helper cell type 1 and 2 response with distinct functions. Targeting of cytokines has been a promising approach within the last years. The clinical and experimental data of IL-10, IL-12 and IL-18 will be described. The anti-TNFα therapy with infliximab will serve as example to outline therapeutic impact as well as difficulties associated with anti-cytokine therapy, in particular infectious complications. Cytokines and other mediators activate second messenger systems which then perpetuate the inflammatory process. MAPK inhibitors targeting these signaling pathways are currently in phase II clinical trials for Crohn's disease. Last, inflammatory cells require the expression of adhesion molecules to allow for cell recruitment, migration, and adhesion, therefore blockade or neutralization of these adhesion molecules seems to be an attractive approach, clinical data are as yet not that conclusive.

The overview provided, does not claim to be complete, however, it may serve to facilitate the understanding of the complex variety of immunomodulatory approaches in IBD (*Fig. 1*).

Immunomodulatory approaches

Antigen processing and presentation

There is evidence that specific microbes in the commensal gut microflora are more important than sporadic infections in atopic disease prevention. Gastrointestinal flora is essential for the maintenance of mucosal tolerance, which is disrupted in patients with inflammatory bowel disease. Several processes seem to be involved in this mechanism: T-helper cell type immunity [1], induction of oral tolerance [2], and IgA production, an essential component of immune defense [3]. Inflammatory bowel disease is a relapsing inflammatory disorder of unknown cause in which genetic, immunological, and environmental factors may be involved [4]. It has also been postulated that ulcerative colitis is at least partly caused by infection because there are common histological features with infectious colitis and it is difficult to induce colonic inflammation in germ-free animals [4].

Probiotics

Probiotics are cultures of potentially beneficial bacteria of healthy gut microflora [5]. Several bacteria have been proven to

express beneficial effects in vivo. Kaliomäki and colleagues could demonstrate that *Lactobacillus GG* was effective in prevention of early atopic disease in children at high risk [6]. In addition, treatment with a non-pathogenic *E. coli* has an equivalent effect to mesalazine in maintaining remission of ulcerative colitis. Furthermore, Gionchetti and colleagues demonstrated that oral administration of a probiotic preparation is effective in preventing flar-ups of chronic pouchitis [7].

Antibiotics

Several studies indicate an efficacy of a combinational therapy of metronidazole plus ciprofloxacin with or without steroids in the treatment of recurrent or refractory pouchitis [8-10]. In addition, patients with Crohn's disease and involvement of the colon might benefit from this combined antibiotic therapy [11].

Granulocyte-macrophage colony stimulating factor

Clinical data

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a growth factor that has been in clinical practice for years in particular in oncology in order to reconstitute the immune system after chemotherapy or bone marrow transplantation. It appears to be a provocative approach to introduce GM-CSF in the treatment of IBD where immunosuppressive strategies have been dominating for decades. In a pilot study performed at the University of Washington, St. Louis, USA, eleven patients with Crohn's disease were treated with GM-CSF. Nine out of 11 treated patients showed a significant decrease in the Crohn's disease activity index (CDAI) [12]. Six out of 11 patients went into remission while treatment with GM-CSF. In one patient a fistula closure was observed. After the GM-CSF treatment period, 2 patients presented with a relapse, however responded again after readministration of GM-CSF [12]. However, these are preliminary data, in order to evaluate efficacy and to be able to compare GM-CSF treatment with standard therapy the results of the planned double-blind multi center trial will provide conclusive data.

Potential way of mechanism

GM-CSF is targeting one of the first steps by supporting potentially weak cell populations in IBD patients, the macrophages and granulocytes [13]. Both are responsible for the unspecific immune defense against invading bacteria. Is this unspecific defense failing, the specific immune response is stimulated and followed by an exacerbation of inflammation. GM-CSF is expressing anti-inflammatory potency by supporting the unspecific immune system and therefore inhibition of the specific immune response. Preliminary data show promising results in patients with IBD, but the results of the phase II trial will have to proof this approach.

T-cell recognition and activation of $CD4^+$ T-cells

The early steps of T-cell activation in the mucosa can be down-regulated in by immunosuppressives such as azathioprine, cyclosporine A, methotrexate or cyclophosphamide.

Azathioprine

Clinical data

The thioprine analogues azathioprine and 6-mercaptopurine have been considered as treatments for Crohn's disease since the initial report of Brooke and colleagues describing healing of fistulas with azathioprine [14]. A decade later the efficacy of this drug was proved in a randomized controlled trial by Present and colleagues [15]. A meta-analysis of studies of azathioprine and 6-mercaptopurine in Crohn's disease has provided the best summary of the effects of these drugs. For active disease, treatment produced an odds ratio of response of 3.09 compared with placebo, with improved response when treatment was continued for at least 17 weeks [16]. Convincing evidence of benefit was also seen in maintenance of remission (odds ratio over placebo 2.27) [16]; glucocorticoid sparing [17, 18], and improvement in fistulas [16]. In addition, a recent study provided important information to the question whether or not one has to discontinue ongoing immunosuppressive therapy with azathioprine/6-mercaptopurine before elective surgery? The study by Aberra and colleagues demonstrated convincingly that azathioprine/6-mercaptopurine alone and the addition of azathioprine/6-mercaptopurine to corticosteroid therapy did not result in a significant increased risk of postoperative infectious complications [19]. Overall, approximately one half to two thirds of patients may respond to therapy. In contrast to glucocorticoids, mucosal healing is frequently seen with adequate dosing of thioprine agents [20].

Mechanisms of action

The molecular mechanism of azathioprine action however has been unresolved. Recent data suggest that azathioprine and its metabolites 6-mercaptopurine as well as 6-thioguanine induce apoptosis in primary intestinal lymphocytes [21]. Additional studies revealed a suppression of NF-κB and the MEK kinase phosphorylation after azathioprine treatment. The authors explain this by binding of the azathioprine-generated 6-Thio-GTP to GTPase Rac1 instead of GTP. This is resulting in an inhibition of the Rac/MEKK/NF-κB pathway and a following caspase-9-dependent apoptosis. The affinity of the 6-Thio-GTP Rac binding is 20-times lower than that of regular GTP which is explaining the delay of therapeutic efficacy well known for azathioprine. In summary, these data suggest that azathioprine is inducing a selective apoptosis of activated T-lymphocytes in the lamina propria and therefore expressing anti-inflammatory potency.

Cyclosporine A

Cyclosporine is being used increasingly in severe ulcerative colitis. Favorable results have been reported for intravenous use (4 mg/kg) and have been confirmed in a placebo-controlled trial [22]. From 50 to 80% of patients with severe attacks who fail to respond to intravenous glucocorticoids may avoid colectomy during the attack. Anecdotally, patients appear to respond well but tend to relapse soon after treatment is stopped. In contrast, there appears to be no significant role for cyclosporine in Crohn's disease. A series of uncontrolled and randomized controlled trials has shown high doses of cyclosporine to be efficacious in treating inflammatory disease and fistulas but an unacceptable high cost in adverse effects [23].

Methotrexate

Clinical data

A promising open-labeled study of methotrexate in IBD [24] led to a randomized controlled trial in Crohn's disease. Patients with chronic active Crohn's disease despite at least 3 months of prednisone 12.5 mg/day or more with at least one failed attempt to taper off treatment were enrolled [25]. Overall, 39.4% of patients assigned to methotrexate achieved remission off prednisone compared with 19.1% of placebo-treated patients [25]. Methotrexate is also beneficial in maintenance of remission. A follow-up study randomized patients who achieved remission on methotrexate 25 mg once weekly i.m. to receive either placebo injections or 15 mg methotrexate i.m. At week 40, 65% of patients treated with methotrexate were still in remission compared with 39% of placebo-treated patients [26]. Treatment was well tolerated.

Experimental data

Although methotrexate is a folate antagonist, the drug is often given with folic acid to prevent nausea and stomatitis. Therefore, other modes of action are likely responsible for its efficacy. The drugs possess a variety of immune-modulating and anti-inflammatory effects, including inhibition of IL-1, IL-2, IL-6 and induction of adenosine, which has direct immunosuppressive properties [27].

Methotrexate may be considered as an alternative to the thiopurine analogs, particularly among patients who do not tolerate these drugs. Some patients who fail to respond to 6-mercaptopurine may respond to methotrexate [28].

Cyclophosphamide

One major problem in the management of steroid refractory attacks of patients with IBD is the establishment of a rapidly acting immunosuppressive regimen. Previous studies investigating the efficacy of cyclophosphamide in Crohn's disease are either single case reports or low dose oral cyclophosphamide instead of intravenous pulse therapy was administered [29,30]. Intravenous cyclophosphamide pulse therapy is well known for its efficacy in vasculitis. Based on these experiences seven steroid-refractory patients, five with Crohn's disease, two with indeterminate colitis, received 4 to 6 cycles of monthly treatment of intravenous cyclophosphamide (750 mg) in a prospective, uncontrolled pilot study [31]. All patients improved after two intravenous pulses of cyclophosphamide and six of seven patients achieved complete remission. Tapering to low dose steroids was possible in all responders. Remission was maintained in all patients for 18 months but required a second course of cyclophosphamide in one patient. The drug was well tolerated, except for two episodes of candida esophagitis. These preliminary results are promising and merit evaluation in a controlled trial [31].

Generation of a Th1/Th2 response

With the targeted use of cytokines attempts have been made to influence the balance between the T-cell subpopulations. IL-10 as well as the blockade of TNFα, IL-12 and IL-18 should be mentioned in this context. The problems which occur when a specific immune mediator is blocked will be discussed in more detail with the available data from the infliximab therapy.

Interleukin-10

Clinical data

IL-10 has first been described in 1989 as anti-inflammatory cytokine and came soon into the focus of interest as potential new anti-inflammatory mediator [32]. Administration of rhIL-10 to healthy volunteers is well tolerated. In higher concentrations a non-clinical significant decrease in hemoglobin as well as platelet count has been described [33]. Because of the promising in vitro and experimental data as further described below, IL-10 was tested as potential therapeutic agent for inflammatory bowel disease, in particular Crohn's disease. However so far, multiple placebo-controlled studies have been performed without proving clinical efficacy. Different subgroups have been studied, for instance: mild to moderately active Crohn's disease (n=95) [34] and therapy refractory Crohn's disease (n=329) [35]. In both studies IL-10 was well tolerated and a tendency towards clinical improvement could be demonstrated but no remission was observed. An additional study by Colombel and colleagues showed that treatment for 12 weeks after intestinal resection was safe and well tolerated, but there was also no evidence of prevention of endoscopic recurrence of Crohn's disease observed [36].

Anti- and pro-inflammatory properties of Interleukin-10

IL-10 as a T-helper cell type 2 cytokine is directly inhibiting several pro-inflammatory mediators known to contribute to the inflammatory process in inflammatory bowel disease [37]. In the last years IL-10 has in addition been described as predominant cytokine of so-called regulatory T-cells [38]. However, there are data from the recent literature suggesting a distinct pro-inflammatory role for IL-10. In particular, when LPS administered to humans followed by an injection

of IL-10, IL-10 enhanced IFNγ release as well as the release of the IFNγ-dependent chemokines IFNγ-inducible protein-10 and the monokine induced by IFNγ [39]. In addition, when administering IL-10 to Crohn's disease patients the serum neopterin concentrations of IL-10-treated patients increased as well as the IFNγ release after post treatment in vitro stimulation [40].

IL-10 and experimental evidence in animal models

Still, experimental models strongly indicate that the local IL-10 concentration at the site of inflammation might represent the crucial factor. For instance, *Lactococcus lactis* secreting IL-10 administered orally to mice prevented dextran sulphate sodium-induced colitis [41]. In addition, the CD4 $CD45Rb^{low}$ cells, the population known to prevent colitis induction by CD4 $CD45Rb^{high}$ cells, is not mediating this protective effect when isolated from IL-10 knockout mice [42]. Therefore current strategies are focusing on technical approaches facilitating an increased IL-10 synthesis at the site of inflammation.

Interleukin-12

Clinical data

IL-12 represents the predominant cytokine responsible for the development of Th1 cells, the T-cell population known to contribute significantly to the inflammatory process in particular in Crohn's disease [43-45]. Currently an antibody against the IL-12 receptor is under investigation in a phase IIa clinical trial in ulcerative colitis patients and in addition, an anti-IL-12 antibody is under investigation in a phase II trial in patients with Crohn's disease.

Experimental data

Early experimental data suggest that the neutralization of IL-12 might results in an amelioration of disease. For instance, administration of monoclonal anti-IL-12 antibodies to mice suffering from trinitrobenzene sulfonic acid-induced colitis led to a striking improvement in both the clinical and histopathological aspects of the disease and frequently abrogated the established colitis completely [46]. In later studies it could be demonstrated that the amelioration of disease severity after anti-IL-12 treatment is associated with an increase in apoptosis at the site of inflammation [47]. An increase in apoptosis and the association with disease improvement has been demonstrated in several experimental models for a variety of mediators [48, 49] and there exists additional evidence in humans that this mechanism may in fact be of significance [50].

Interleukin-18

Clinical evidence

Several studies provide strong direct and indirect evidence for a significant role of IL-18 in intestinal inflammation. Consistent with an increased Th1 response in Crohn's disease, several groups could independently demonstrate a significant up-regulation of IL-18 expression in the inflamed lesions of the intestine, mostly localized to macrophages and epithelial cells [51,52]. This is of particular interest mainly because no up-regulation of IL-18 was observed in patients with active ulcerative colitis or healthy controls. While increased IL-18 expression is providing first evidence for a possible role in disease, in order to prove that IL-18 participates in the inflammatory process of intestinal inflammation it has to be demonstrated that blockade of IL-18 results in amelioration of disease severity.

Experimental data

In the recent literature, four studies using different animal models of colitis and different ways of IL-18 neutralization approach this question. Convincingly, all groups demonstrate a therapeutic efficacy for all anti-IL-18 strategies employed independently from the model investigated [53-56]. Results from these experimental models, in combination with the descriptive data from patients with Crohn's disease, suggest an important function of IL-18. IL-18 requires the cleavage of the interleukin-1β converting enzyme (ICE) in order to become activated [57]. Recent studies examined the acute and chronic model of DSS-induced colitis in ICE knockout mice [49]. In particular, during chronic administration of DSS, ICE knockout mice presented with an almost complete absence of colitis. Several ICE inhibitors are available for experimental use. For instance, Pralnacasan is currently in phase II trials in rheumatoid arthritis. No toxic side effects have been observed [58]. Compared to currently used strategies to suppress specific cytokines, which mostly implicate antibody therapy, the possibility of having an orally available drug whose half-life can be easily controlled is highly intriguing.

Tumor necrosis factor-α

Clinical studies

The TNFα antibody infliximab has proved to be effective in certain clinical situations in Crohn's disease. Targan and colleagues could first demonstrate in a multicenter, double blind, placebo-controlled trial over 12 weeks, the efficacy of infliximab in patients with moderate-to-severe, treatment-resistant Crohn's disease [59]. 48% of patients were in remission 4 weeks after single 5 mg/kg BW infusion compared to 4% in the placebo groups [59]. Interestingly, administration of 5 mg/kg BW was clearly superior to 10 or 20 mg/kg BW treatment. D'Haens and colleagues subsequently investigated the endoscopic scores after treatment with 5, 10 or 20 mg/kg BW infliximab [60]. The Crohn's disease endoscopic index of severity decreased significantly, 62%, 68% and 21% in the 5 mg/kg BW, 10 mg/kg BW and the placebo group, respectively [60]. The clinical improvement was accompanied by significant healing of the endoscopic lesions and a disappearance of the mucosal inflammatory infiltrates. In the recently published ACCENT I trial, Hanauer and colleagues evaluated the long-term efficacy of repeated infliximab administration [61]. Repeated infusions of either placebo, 5 mg/kg or 10 mg/kg BW, at 2, 4, 6 and then every 8 weeks were administered. 335 out of 580 patients showed a primary response. These results indicate that patients with Crohn's disease who respond to an initial dose of

Table 1. **International recommendations for infliximab therapy in Crohn's disease (CD)**

Target patient group	• Efficacy has been established in active CD, we recommend restriction to its use to refractory CD • Refractory = full and adequate dosed course of glucocorticoids in addition to immunomodulation has failed or other drugs are not tolerated or not appropriate and surgery is not indicated • First-Line use in non-fistulizing, uncomplicated CD is not recommended • Infliximab should be part of a long-term strategy
Administration	• Maximum of two infusions of infliximab within 4 weeks • Clinical benefits are of limited duration • Readministration is warranted in patients who relapse under adequate immunosuppressives

infliximab are more likely to be in clinical remission at week 30 and 54, to discontinue corticosteroids, and to maintain their response for a longer period of time, if infliximab treatment is maintained every 8 weeks [61]. Repeated infusions have been demonstrated to be associated with an increased risk of infusion reaction. A recent study by Baert and colleagues could confirm these findings and were able to provide evidence that the development of antibodies against infliximab is in addition associated with a reduced duration of response to treatment. In addition, the data from this study are indicating that concomitant immunosuppressive therapy reduces the magnitude of the immunogenic response [62].

However, it has become evident at the same time that such treatments are combined with corresponding risks: for instance, severe mycobacterial infections were observed in treated patients [63] and are discussed in more detail below.

Mystery of etanercept failure in the treatment of Crohn's disease

Etanercept is a construct of two identical extracellular chains of the soluble TNF-RII (also known as the p75 receptor) linked to the Fc domain of IgG1. It has been demonstrated to be highly effective in the treatment of rheumatoid arthritis [64]. Hence, it was first obvious to assume that etanercept administration would also prove to be effective in Crohn's disease patients. Sandborn and colleagues performed an eight-week placebo-controlled trial in 43 patients with moderate to severe active Crohn's disease. Etanercept was injected twice weekly in a concentration of 25 mg, the concentration which has been shown to be of therapeutic impact in patients with rheumatoid arthritis [64]. After 4 weeks 39% of etanercept-treated versus 45% of placebo-treated patients presented with a clinical response [65]. The authors concluded at this point that administration is safe but not effective and that higher doses may be required to attain a response in patients with active Crohn's disease. However, one might speculate whether or not the low dose or a different mechanism is responsible for the observed difference between etanercept and infliximab. Van Deventer suggests in the Editorial to this study that infliximab but not eternacept can induce apoptosis by binding to membrane-bound TNFα, thereby expressing its ant-inflammatory potency [66]. Since apoptosis induction seems to be crucial in order to achieve clinical improvement this might in fact represent the crucial difference in between the two therapeutic strategies.

Blockade of tumor necrosis factor-α and tuberculosis

Clinical studies

Infliximab is a humanized antibody against TNFα that is used in the treatment of Crohn's disease and rheumatoid arthritis. Approximately 147 000 patients throughout the world have received infliximab [63]. The half-life of infliximab is 10 days [67], and its biologic effect persists for up to 2 months. Keane and colleagues evaluated in a recent study the clinical pattern of disease and the interval between the initiation of infliximab therapy and the onset of disease in 70 reported cases of patients treated with infliximab [63]. Excess TNFα in association with TBC may cause weight loss and night sweats, yet in animal models it has a protective role in the host response to tuberculosis. There is no direct evidence of a protective role of TNFα in patients with TBC. The data by Keane and colleagues, although passive surveillance data are often insufficient to prove a causal relation between an adverse event and a drug, strongly suggest that the association observed is not coincidental. In particular, the pattern of TBC disease was unusual, the majority of patients had extrapulmonary TBC, and 24% had disseminated disease – forms of TBC that are associated with marked immunosuppression. In contrast, among cases of TBC that are not associated with HIV infection, approximately 18% are manifested as extrapulmonary disease, and disseminated disease accounts for less than 2% [68]. Although there is no complete information about the status of these patients with respect to TBC infection before they received infliximab, it is likely that most patients had a reactivation disease. Given the key role of TNFα in the innate immune response to TBC, patients receiving infliximab are probably also susceptible to disease after primary infection and exogenous reinfection with M. tuberculosis.

Consequences

As a consequence of the clinical and experimental data the European Agency for the Evaluation of Medicinal Products (EMEA) provided a public statement on infliximab with an update on safety concerns. The Committee for Proprietary Medicinal Products (CPMP) concluded that infliximab continues to have a positive benefit/risk balance in both Crohn's disease and rheumatoid arthritis, provided that specific changes to the product information restricting the indications in Crohn's disease and reinforcing the special precautions and special warnings have been made. Therefore, because of safety

concerns, the indications for treatment of Crohn's disease have been restricted as summarized in *Tab. 1* [67].

Cell recruitment, migration, and adhesion

Clinical data

In a multicenter, placebo-controlled trial conducted in 75 patients with steroid refractory Crohn's disease subcutaneous treatment with ISIS-2302, an antisense oligonucleotide directed against intercellular adhesion molecule-1 (ICAM-1), did not prove clinical efficacy based on primary endpoints [69]. Positive trends were observed in some of the secondary endpoints [69]. In agreement with these data, the second double-blind, placebo-controlled trial in active steroid-dependent Crohn's disease also failed to demonstrate efficacy [70].

Experimental data

However, the concept of blocking adhesion of inflammatory cells thereby preventing intestinal inflammation is intriguing and there are a variety of animal studies using different models and technical approaches for inhibition of adhesion molecules, supporting this target. While the efficacy of antisense strategies against ICAM-1 could be proven in the model HLA-B27/beta2 microglobuline transgenic rat model, this approach is questionable since no therapeutic benefit could be observed in human trials [71]. More detailed animal studies in the SAMP-1/Yit adoptive transfer model of Crohn's disease in mice could distinguish between an early acute phase and chronic phase of inflammation. These studies suggest that blocking of either ICAM-1 or VCAM-1, in this case by neutralizing antibodies, may have therapeutic benefit for the acute inflammatory component of Crohn's disease [72].

Future studies will have to prove whether for distinct indications this target might be of therapeutic significance.

Second messenger systems

Clinical data

A further possibility of intervention is the down-regulation of "second-messenger systems" which are significant for inflammatory immune reactions. Colonic biopsies from patients with Crohn's disease displayed enhanced JNK and p38 MAPK activation [73]. In a pilot study, 12 patients with severe Crohn's disease received the MAPK inhibitor (CNI-1493) for a total of 12 days. Treatment resulted in diminished JNK phosphorylation and tumor necrosis factor production as well as significant clinical benefit and rapid endoscopic ulcer healing. A clinical response was seen in 67% at 4 weeks and 58% at 8 weeks. Therefore, inhibition of MAPKs provides a novel therapeutic strategy [73]. The efficacy has to be confirmed in the phase II trials.

Experimental data

Phosphorylation of intracellular molecules by a protein kinase called mitogen-activated protein kinase (MAPK) with a molecular weight of 38 kDa plays a major role in the synthesis and activity of several proinflammatory cytokines, particularly IL-1β, TNFα, and the chemokines. Inhibitors of MAPK are non-specific anti-inflammatory agents. P38-MAPK is referred to as a stress kinase because it becomes activated (phosphorylated) in response to extracellular stresse such hyperthermia, hyperosmolarity, ultraviolet light, and radiation. Of major importance is the fact that this kinase, which contributes to the activity of IL-1β and TNFα, is itself activated by IL-1 and TNF. Therefore, phosphorylation of p38-MAPK can occur both via highly evolved, specific cytokine receptor signaling pathways and by the primitive and environmental stress responses common to all organisms. The mode of action of p38-MAPK inhibitors appears to be predominantly through suppression of pro-inflammatory cytokine production. In healthy human volunteers injected with endotoxin, the signs and symptoms of this systemic inflammatory response model were assessed following a single oral dose of a p38-MAPK inhibitor. No fever was observed, and peak circulating levels of TNFα, IL-6, and IL-8 were reduced by 90% [74].

Conclusions

This review provides an overview of the increasing variety of therapeutic approaches in IBD based on the pathogenesis. The advances in the last years in the understanding of mucosal immunology contributed significantly to the development of targeted therapies. Primary goals of therapy are to induce and maintain remission, thus ameliorating symptoms and improving the patient's quality of life. Summarizing all the different therapies discussed above, one has to conclude that, although specific treatments provide more insight in the understanding of disease, the general immunosuppressive drugs such as azathioprine appear to express the most efficacious balance between achieving the therapeutic goals and reducing side-effects. Distinct clinical situations may benefit from more specific therapies, for instance, infliximab treatment in therapy refractory fistulas. However, these more targeted drugs seem to be associated with a yet unknown spectrum of side-effects. Therefore, it is essential to consider the adverse consequences of therapy, particularly with regard to any durable consequences of short-term treatment and adverse effects of maintenance therapy.

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Fig. 1 was reprinted from Gastroenterology 118 (2 Suppl 1), Sands BE, "Therapy of inflammatory bowel disease", 68-82, Copyright (2000), with permission from American Gastroenterological Association.

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Inflammatory response, reactive oxygen species, programmed (necrotic-like and apoptotic) cell death and cancer

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Abstract

In this short review we attempt to establish and/or strengthen connections between clinical, inflammatory manifestation of cancer, inflammatory processes driven by lipoxy-metabolites and their contribution to immortalized phenotype and apoptosis inhibition. Particularly the resemblance between symptoms of inflammation and signs associated with cancer chemotherapy and/or cytokine therapy is illustrated. In this context the role of apoptosis and necrosis in inflammation as well as the role of RedOx processes and lipid-oxidizing enzymes particularly cyclooxygenase-2 (COX-2) and also to lesser extend the 5-lipooxygenase (5-LOX) is highlighted. The multitude of biological effects of reactive oxygen species is shortly summarized and some aspects of it are being discussed in greater detail. Apoptotic cell death is discussed in the context of the "resolve-phase" of an inflammatory response. The disturbance of apoptosis is mainly deliberated in the framework of insufficient removal of immuno-effector cells that may cause autoimmunity. The role of COX-2 in apoptosis resistance is being highlighted mainly in the context of malignant transformation. The mechanism of cell death (apoptotic or necrotic) and its influence on the immune system and potential benefits of necrotic cell death induction during cancer chemotherapy is indicated.

Key words: apoptosis, caspases, cancer, COX-2, inflammation.

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Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; DR5, death receptor 5; GSH, L-γ-glutamyl-L-cysteinyl glycine; 12-HETE, 12-hydroxyeicosatetraenoic acid; HPETE, hydroxyeicosatetraenoic acid; iNOS, inducible nitric oxide synthase; 5-LOX, 5-lipooxygenase; LPS, lipopolysaccharide; LXA4, lipoxin-A4; NSAIDs, nonsteroidal anti-inflammatory drugs; ROI, reactive oxygen intermediates; PGE2, prostaglandin-E2; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; UV, ultraviolet radiation; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

Introduction

Reactive oxygen species (ROS) are associated with the inflammatory response and frequently they contribute to the tissue damaging effects of inflammatory reactions [1-3]. On the other hand they are important mediators of programmed cell death induced by TNF [4,5]. Moreover, in some experimental models, when applied at low concentration they are capable in stimulating cell growth [6,7]. Furthermore, at intermediate concentrations ROS induce apoptosis whereas at higher concentrations it induce necrotic cell death [8,9]. The mutagenic effect of ROS is well established. It contributes to DNA damage evoked by ionizing radiation and certain chemical cycling oxidants (eg. doxorubicin [10] or paraquat, [11]), and under certain circumstances it may lead to cancer.

Inflammation, which was recognized as a simple allergic reaction for decades, is currently being considered to underline pathophysiology of a much broader spectrum of diseases than previously expected. The complex interplay of cellular and humoral mediators during inflammation is unfolding but our understanding of the inflammatory reaction is still incomplete [12,13]. Viruses, frequent pathogenic inducers of inflammatory response, have acquired a number of elaborated ways to evade both, the apoptotic response and inflammatory processes [14,15].

The inflammatory process initiated in response to a pathogen or an injury is maintained at a certain, adequate level till the offending stimulus is neutralized, after which the reaction resolves on its own. In an auto-immune disorder, a harmless antigen is mistaken by the immune system as begin foreign, thus initiating the inflammatory reaction. The persistence of a stimulus, that physiologically resides in the organism, prevents the natural, resolving mechanisms from prevailing [16]. As a result one's own defense mechanism can turn into a perpetuator of a persistent injury which although not fatal, can lead to loss of function of the organs involved.

Additionally, many of the mediators can leak from the local region and initiate inflammatory reactions elsewhere [17]. Animal experiments have been extremely useful in understanding the entire inflammatory reaction since they demonstrate a complete window of events, from the time when the stimulus is given till the reaction naturally resolves [18,19].

ROS and inflammation

ROS formation and degradation are key components of the metabolism of aerobic organisms. Certain levels of ROS are required for normal cell functions, but if in surplus, they will cause oxidative stress [6,20,21]. ROS like superoxide, hydrogen peroxide and lipid hydroperoxides can regulate the activities of several kinases, transcription factors, cell death machinery and proteins such as COX-2 and iNOS [21-24]. ROS also function as second messengers in intracellular signal transduction pathways [6,20,25]. However, upon prolonged activation in vivo, the deleterious effects of ROS and enzymes take an upper hand in the destruction of the tissues by affecting the structure-function model of all macromolecules, sometimes irreversibly. ROS play a major role in the joint destruction by their ability to transform proteins to autoantigens and/or increase the susceptibility of proteins to degradation. Neutrophils play a crucial role in the development and manifestation of inflammation and they are the major source of free radicals at the site of inflammation. Lipid peroxidation mediated by free radicals might yield a large number of reactive aldehydes and also lipid peroxides which are causally involved in pathophysiological changes associated with oxidative stress in cells and tissues [26,27]. GSH (L-γ- glutamyl-L-cysteinyl glycine) is an ubiquitous thiol-containing tripeptide, which plays a key role in cell biology. It is implicated in the cellular defense against xenobiotics and naturally occurring deleterious compounds, such as free radicals and hydroperoxides. GSH status is a highly sensitive indicator of cell functionality and viability [28,29]. RedOx status with increased GSSG formation is a key factor that mediates apoptosis in neutrophils and macrophages [30,31]. The increased level of GSSG concentration point to the prevailing oxidative stress and indicates ongoing ROI detoxification. The overwhelming level of intracellular ROI and GSSG indicates a disturbance in the RedOx status of the cell, a condition that may be followed by apoptosis. Many of the agents capable of inducing ROS, such as intermediate concentration of H202, UV light and ionizing radiation [9,21,32] are also known to evoke apoptosis.

Cyclooxygenases are key enzymes in the prostaglandin synthesis. The inducible isoenzyme COX-2 plays a pivotal role as a mediator of inflammation. COX-2 enzyme activity itself is sensitive to, and regulated by the RedOx status of the environment [33]. Antioxidants inhibit the expression of COX-2 in human alveolar macrophages [34]. One regulatory element that can control the COX-2 expression is NF-κB activity. NF-κB and AP-1 transcription factors are activated by changes in the RedOx status of the cell [23]. The AP-1 subunits, c-fos and c-jun, are regulated by oxidant response elements and are induced by lipopolysaccharide (LPS).

Oxidized phospholipids were shown to inhibit COX-2 expression induced by LPS in macrophages by interfering with the NF-κB/lκB, MAP kinase and ERK2 pathways in atherosclerotic lesions [35]. Based on the above data, it can be hypothesized that conditions of oxidative stress/antioxidant imbalance impair the capacity of macrophages to correctly contribute to the resolution of the inflammatory processes. The inhibition of the COX pathway increases formation of ROS through peroxidative cleavage of 5-hydroxyeicosatetraenoic acid (5-HPETE), and hence 5-LOX inhibitors would attenuate this effect. The present study demonstrated the effectiveness of anti-oxidants in reducing the inflammatory reaction, expression of COX-2, iNOS levels and oxidative stress by inducing apoptosis in the inflammatory cells. These studies thus indicate, the importance of ROS in bringing about the inflammatory reaction and also the therapeutic potential of anti-oxidant mixture in reducing inflammation. Several attempts have been made to turn naturally occurring antioxidants into anti-inflammatory agents [36-40], but their successful clinical implementation was infrequent. The relative lack of effectiveness of this approach may be related to the fact that inhibition of the expression of genes involved in inflammatory response alone may be insufficient to fully block the process. Apoptotic mechanisms that remove superfluous immunocompetent cells need to be in place as well (see below).

p53 and inflammation

Transcriptional activation of p53 in the area of inflammation is probably induced in response to the toxic environment of the inflamed tissue. The local production of oxygen radicals, nitric oxide, cytokines, eicozanoids etc. may lead to the accumulation of normal p53 [41]. The overexpression of wild type p53 in inflammatory disorders such as ulcerative colitis and rheumatoid arthritis [42], cancers [43], autoimmune encephalomyelitis [44] and various animal models of chronic inflammation [45] have been well documented. The central involvement of p53 in initiating apoptosis after exposure to a variety of cellular stress inducers is presently becoming established [46,47]. The increased p53 levels and augmented apoptotic cell death is observed mainly in the resolution of inflammation [48]. The inhibition of p53-dependent apoptotic mechanisms in inflammatory effector cells may contribute to the transition of the acute inflammatory response into the chronic phase of inflammation. Mediators of inflammatory response may influence the expression level of p53. For example, p53 expression was reduced in the presence of COX-2 inhibitor celecoxib, thus p53 may be

directly regulated by inflammatory mediators. On the other hand, COX-2 itself has been reported to regulate the expression of p53 and in turn it is regulated by NF-κB, a transcription factor that is well known for modulation of expression of inflammatory molecules [49,50] in vitro.

The role of apoptosis in inflammation

In order to cease the inflammatory response the infiltration of inflammatory effector cells has to be stopped and the existing population of pro-inflammatory cells must be eliminated without provoking the release of pro-inflammatory mediators [51]. This is achieved by the apoptotic process that normally involve activation of the proteolytic cascade of caspase family proteases [52-54]. An important determinant of the resolution of inflammation is apoptotic removal of leukocytes with subsequent clearance of the apoptotic bodies by phagocytosis [55,56]. Recent publications also show active suppression of pro-inflammatory cytokine production during phagocytosis of apoptotic cells (reviewed in [56]). Therefore, the development of therapeutic strategies aimed at inducing apoptosis in rheumatoid arthritis and other chronic inflammatory disorders is an attractive goal since (I) reduced apoptosis may play an important role in the pathogenesis of chronic inflammation [57], and (II) promotion of apoptosis in the chronically inflamed tissue may have an anti-inflammatory effect by itself. This goal can be addressed in two ways: (1) by inducing apoptosis in the inflammatory effector cells, or (2) by inhibiting the anti-apoptotic mechanisms of these cells [58,59]. Reduced apoptosis of synovial cells has been described in residential synoviocytes as well as in inflammatory cells that are associated with the pathogenesis of rheumatoid arthritis [60,61]. Although a detailed understanding of mechanisms that prevent synovial fibroblasts from programmed cell death is lacking, several anti-apoptotic molecules have been identified. Among them, transcriptional regulators such as p53, NF-κB and Stat3, have been suggested to regulate apoptosis most prominently [49,62]. The increased apoptosis of immune effector cells in the resolution-phase of inflammatory response is associated with the increased expression of Apaf-1, the key component of apoptosome. Unlike during tissue remodeling for example, when the surrounding cells remove the bulk of dying neighbor cells, macrophages are the main cells that phagocytose apoptotic immune-effector cells during downscaling of the specific immune response. Phagocytosis triggers macrophage release of CD178 (Fas ligand), an event that may lead to the induction of the apoptosis of bystander leukocytes [63]. This will happen only towards the end of a successful inflammatory response since freshly stimulated T-cells are resistant towards CD95(Fas, Apo-1)-triggered apoptotic death [64,65]. Prolonged exposure of cultured human monocyte-derived macrophages to a cytokine cocktail including, GM-CSV, TNF, IFN-γ, IL-1β, IL-10, enhances their capacity to phagocytose apoptotic cells in vitro [66] suggesting that this process is dynamically regulated at the site of inflammation. LPS, a potent cofactor of macrophage activation in vitro, prevented apoptosis in human peripheral blood monocytes, allowing their maturation into macrophages [67]. Although the mechanism by which LPS promotes viability of monocytes is not clear, macrophage phagocytosis of apoptotic cells is accelerated by the endogenous lipooxy-metabolites like lipoxin-A4 (LXA4), [68] 12-hydroxyeicosatetraenoic acid (12-HETE) and prostaglandin-E2 (PGE2) [69].

COX-2 activity is an important component of all inflammatory reactions. Although there are no reports of COX-2-specific effects on apoptosis during inflammation, both isoforms of COX are known to have a significant role in apoptosis and cell survival as it has been observed in studies on cancer [70,71]. Furthermore, a higher number of apoptotic neutrophils was observed in COX-2 deficient mice rather than in COX-1 deficient mice [72] suggesting an anti-apoptotic role for COX-2. Overexpression of COX-2 has been linked to down regulation of apoptosis and thus to facilitation of malignant transformation in many cell types [73]. Thus, while the COX-2 – apoptosis link seems to be a normal physiological process, there are multiple lines of evidence indicating that inappropriate up-regulation of COX-2 prolongs the survival of malignant cells and leads to phenotypic changes associated with metastatic potential [61]. Overexpression of COX-2 inhibits death receptor 5 (DR5) expression and confers resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in human colon cancer cells [74].

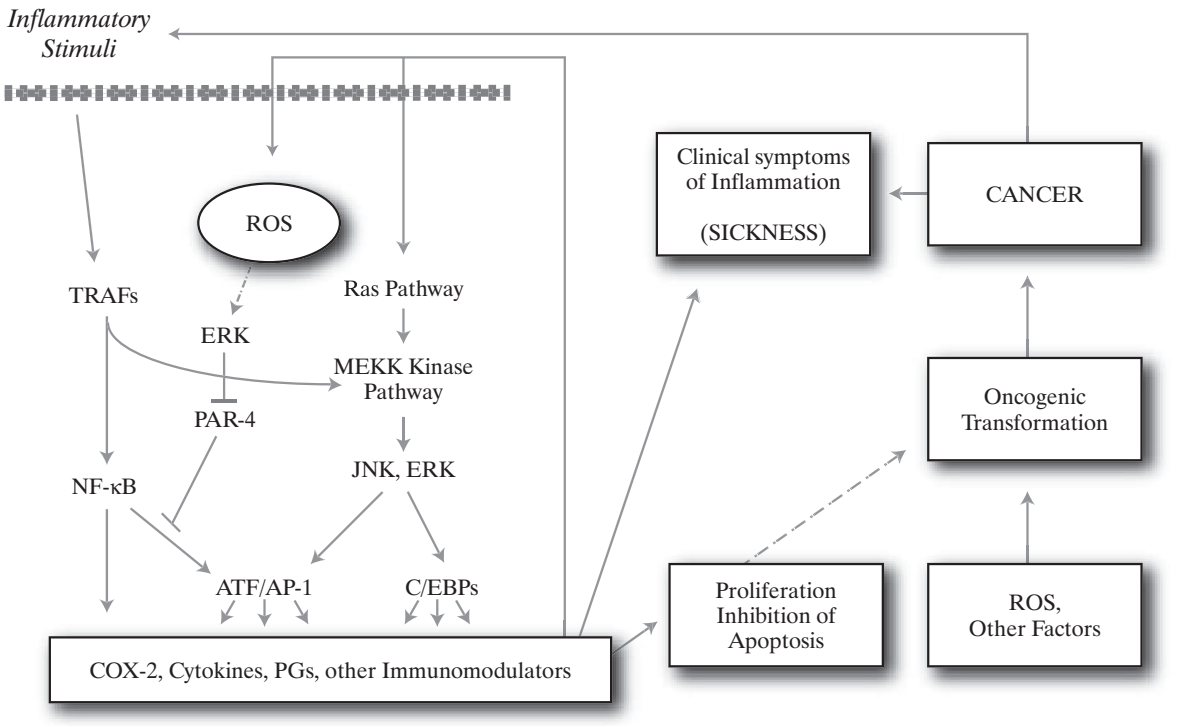
Accumulation of arachidonic acid (AA) activates sphingomyelinase activity, leading to production of apoptotic-inducer, ceramide. This accumulation has been shown both: 2 and you have 3 in primary fibroblasts, immortalized keratinocytes and epidermal cancers [75]. Celecoxib, a COX-2 inhibitor, induced apoptosis in various models of inflammation studied as well as in some experimental tumor models [76,77]. This pro-apoptotic action of celecoxib was shown to be COX-2 dependent [78] as well as independent [79,80]. However this observation also explains the reason why celecoxib is such a good anti-inflammatory agent, as it not only reduces the levels of PGE2, but also kills the inflammatory cells.

Cancer and inflammation

Inflammatory symptoms accompany malignant proliferatory diseases

Cancer patients frequently suffer from symptoms resembling an inflammatory response, resulting from the primary disease and/or from the treatment (*Fig. 1*). The symptoms include pain, gastrointestinal problems (e.g. nausea, diarrhea), wasting/ /cachexia, fatigue, cognitive impairments, anxiety, and depression. Symptoms can cause treatment delays or lead to premature treatment termination. It also may impair function, and rehabilitation, and cause significant distress to the patient [81,82]. Mechanisms related to stress/immune response may underlie or contribute to at least some of those symptoms [82,83]. This has been demonstrated in animal models of sickness behavior, which share symptoms with those patients with cancer. Sickness behavior refers to a constellation of physiologic and behavioral responses observed in animals after the administration of inflammatory agents or specific pro-inflammatory cytokines [84-88]. Sickness behavior can be elicited in animal models by bacterial infections and by administration of pathologic components

Figure 1. **Interplay between inflammatory stimuli, signaling pathways, and ROS, – effects on oncogenesis and clinical symptoms (sickness). The schema outlines signaling pathways involved in inflammation and it provides relation between inflammatory response, proliferation, inhibition of apoptosis and oncogenic transformation. The depicted signaling pathways synthesize the information provided in the main text. Some kinase signaling pathways have been adapted from the literature [155]**



of bacteria such as LPS. Physiologic components of sickness behavior are similar to these observed by cancer patients and they include acute-phase responses (fever, systemic depletion of body electrolites), pain (hyperalgesia), wasting, increased activity of the hypothalamic-pituitary-adrenal (HPA) axis and autonomic nervous system [85]. Behavioral components include a general decrease in activity, somnolence, cognitive impairment (impaired learning), decreased social interaction and exploration, decreased sexual activity, and decreased eating [89]. The responses characteristic of sickness behavior can also be elicited by systemic administration of pro-inflammatory cytokines including IL-1β, TNF, IFN-γ, and IL-2, administered subcutaneously, intravenously, or intraperitoneally [84-88,90--96]. Pro-inflammatory cytokines play a central role in preclinical models that focus more specifically on the symptom of peripheral neuropathic pain/hyperalgesia (hypersensitivity to cutaneous stimuli). Experimental animals, like humans, develop peripheral neuropathic pain following treatment with the chemotherapeutic agents commonly used to treat cancer, like vinca alkaloids, taxanes, and cisplatin [97-99]. In both humans [100] and animals, [101] exposure to γ-irradiation also often produces neuropathic pain-like syndromes and insensitivity to the analgesic properties of morphine. Peripheral neuropathy could be due, at least in part, to the induction of proinflammatory cytokines around nerve endings. For example, production by immune cells and/or cancer cells of the proinflammatory cytokines IL-1 β, IFN-γ, and TNF can be increased by exposure to paclitaxel, [102,103] cisplatin, [104] or irradiation [105].

Clinical evidence consistent with the role of inflammatory cytokines in symptom occurrence by cancer patients

The hypothesis that cytokines may play a mechanistic role in cancer-related symptoms is consistent with various clinical observations. Non-cancer patients who received cytokine therapy displayed many of the symptoms that are observed in cancer patients. For example, patients with hepatitis C virus infection and those with the acquired immunodeficiency syndrome (AIDS) who received IFN-α therapy endured symptoms of pain, fatigue, cognitive impairment, psychosis, and depression [106,107]. A similar symptom profile was observed among patients with renal cell carcinoma, chronic myelogenous leukemia, melanoma, all of whom received IFN-γ, IL-2, or IFN-α plus IL-2 [108-112]. Concurrent administration of oral dexamethasone (an immunosuppressant) and high-dose IFN-α significantly reduced the occurrence of influenza-like symptoms and fatigue in patients with advanced renal cell carcinoma [109]. Interleukin-6 induced fatigue, inactivity, and poor concentration when administered to normal subjects [113]. Neuropathic pain is a frequent complication of chemotherapy with vinca alkaloids, taxanes, and cisplatin and often persists long after treatment has ended [114]. Psychophysical studies of chemotherapy-induced

neuropathic pain demonstrated multiple zones of sensory disturbance similar to those observed in cancer and non-cancer patients experiencing pain after cytokine therapy [106,107]. Other evidence suggested that fatigue is associated with elevations in such proinflammatory cytokines as IL-1β, IL-6, TNF, and IFNs [112]. Cell types that are potential sources of cytokines and other immunoregulatory factors in cancer patients include the cancer cells themselves, [103] immune cells (neutrophils, macrophages, lymphocytes), [87,88,102,104,105] and nervous system cells (paraganglial cells, astrocytes, oligodendrocytes, microglia, and Schwann cells) [87,115,116].

Arachidonic acid metabolites and cancer

Connections between cancer and inflammation are not restricted to overlapping clinical (inflammatory) symptoms but they have much deeper, molecular foundations. For example, several reports indicate that COX-2 and 5-LOX expression may be associated with carcinogenesis most likely due to its apoptosis modulating properties [117,118]. COX-2 was shown to be upregulated in a variety of human cancers including colon, gastric, esophagus, pancreas and breast cancer, while undetectable in most normal tissues [119-124]. Furthermore, over-expression of COX-2 was sufficient to cause tumorigenesis in animal models and to render cells resistant to apoptotic stimuli [125-127].

Supporting evidence for a crucial role of COX-2 in cell survival was also provided in a COX-2 gene knock-out experiment that was done in a genetically driven mouse model of intestinal carcinogenesis. Lack of COX-2 resulted in substantial reduction in the number and size of intestinal polyps [128]. Together, these findings suggested that inhibition of the COX-2 activity, and hence the resulting decrease in prostaglandin production may contribute to the previously described anticancer effect of nonsteroidal anti-inflammatory drugs (NSAIDs) [129-131]. In addition, selective COX-2 inhibitors have been demonstrated to modulate tumorigenic, angiogenic and apoptotic events resulting in reduction of tumor incidence and progression [127].

The specific COX-2 inhibitor NS-398, for example, was shown to suppress tumor growth of different cancer cell lines and to induce apoptosis in human colon carcinoma, prostate carcinoma and esophageal adenocarcinoma cells [132-134]. Other specific COX-2 inhibitors such as nimesulide and celecoxib induced apoptosis in non-small lung cancer, prostate carcinoma, as well as colon cancer cells [135-137], and efficiently inhibited tumor growth in animal models, respectively [138]. COX-2 has therefore potential to become an attractive target for the development of novel anti-cancer strategies.

Synopsis

This short review was not designed to fully summarize our knowledge on the issues in scope (given the broadness of the topics) but rather its intention was to provide new looks and strengthen connections between inflammation, cancer, programmed (apoptotic and necrotic-like) cell death and oxidative stress, with the hope that the integrated approach will fruit development of new anticancer strategies. The interplay between inflammation, oxidative stress, apoptosis and cancer is already, to a certain extent, mirrored by the attempts of pharmacologic industry to cross-apply our current knowledge from these fields to develop new therapy approaches. For instance, strategies that modulate cellular RedOx potential are being applied to restraint inflammation (antioxidants) [139,140] or to kill cancer cells (prooxidants, e.g. carmustine, doxorubicin, paraquat) [10,141-144]. Another application of trans-disciplinary research is a trend to tap into our recent knowledge on programmed cell death for the development of novel therapies for stroke, myocardial infarction or acute and chronic inflammatory diseases [145-147].

Activation of apoptosis or attenuation of resistance of transformed cells towards cell death induction is perhaps the most promising direction chosen by many pharmaceutical companies to develop new anti-cancer drugs [148-150]. The knowledge of apoptosis can yet be used in another way in cancer therapy, eg. for cryo-conservation of hematopoetic stem cells commonly use in aggressive chemotherapy protocols [151]. As the last decade's "hype" about apoptosis research is cooling down, and our understanding of the process is mounting, more and more researchers look into modulation of cell death program so that a mixture between apoptosis and necrosis can be induced [4,5,152]. This approach has a significant therapeutic potential since necrotically-dying cells release their protein content into the surrounding tissue, thus making mutated proteins accessible for antigen presentation, recognition and (hopefully) activation of tumor-specific immune response. Induction of controlled necrosis in tumor cells, or better a mixture of necrosis and apoptosis, would have an activatory effect on the immune system [153,154]. The released tumor fragments would certainly attract the attention of primary- and adaptive immunocompetent cells, thereby contributing to the "bystander effect" and clearance of transformed cells. There is an additional optimistic aspect to of all that. Tumor cells can only become dangerous if they successfully evade both necrotic and apoptotic pathways, so reactivation of one of them may suffice to control the malignancy.

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Fibrogenesis in the pancreas

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Abstract

In recent years, numerous studies have provided novel insights into the pathomechanisms of pancreatic fibrogenesis. This includes in particular the identification and characterization of the pancreatic stellate cells (PSCs) and their role in the synthesis of extracellular matrix (ECM) proteins. It has become clear that pancreatic stellate cell activation is regulated by a complex network of growth factors and cytokines and results in increased expression and release of collagens I and II, fibronectin and other components of ECM. Among the cytokines involved in PSC activation and other fundamental mechanisms of pancreatic fibrosis, transforming growth factor beta (TGF β) is of particular relevance. TGF β stimulates PSC activation and induces transcription of ECM proteins mainly via activation of the Smad proteins which regulate gene expression through functional interaction with co-operating partner proteins such as the zinc finger transcription factor Sp1. Recent progress in understanding of the biochemical and molecular mechanisms of pancreatic fibrosis, is reviewed here.

Key words: pancreas, fibrosis, pancreatic stellate cells, TGF ß, extracellular matrix.

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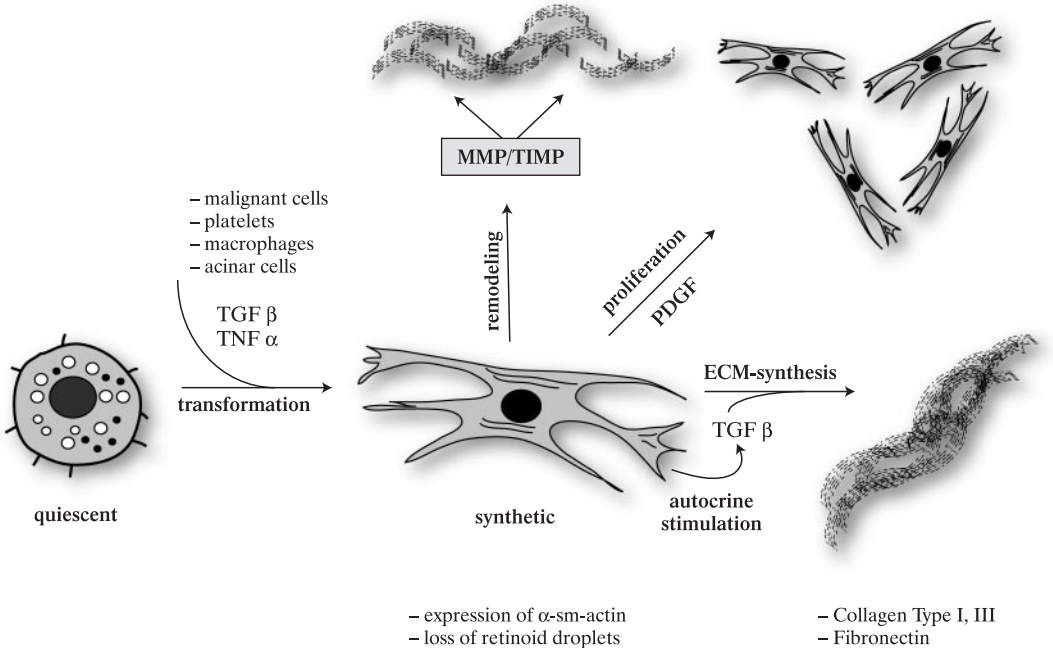
Introduction

Pancreatic cancer, chronic pancreatitis and acute pancreatitis are characterized by profound alterations of extracellular matrix (ECM) formation and composition [1-3]. In these pancreatic diseases the appearance of fibrotic tissue is a result of increased deposition and reduced degradation of extracellular matrix.

The extracellular matrix (ECM) is comprised of four major classes of macromolecules – the collagens, proteoglycans, structural glycoproteins, and elastin [4-6]. Individual members of each class and family of ECM molecules were found to exhibit a degree of tissue-specific distribution implicating the matrix in development and tissue function [7,8]. Cell surface receptors for individual ECM components were identified, which provided a rational basis for linking the ECM with the cell [9-11]. From these discoveries it is now evident that the extracellular matrix is composed of a number of different macromolecules whose structural integrity and functional composition are important in maintaining normal tissue architecture, in development and in tissue-specific function [4,6,8]. On the other hand, it has been recognized that dysfunctional matrix components and abnormalities in ECM biosynthesis and catabolism are of importance in both inherited and acquired diseases and in normal wound healing [4-6].

Years ago, we and others identified "fibroblast-like" cells in the pancreas that show characteristics of myofibroblasts, e.g. expression of α-smooth muscle actin, synthesis of collagens and fibronectin, and the formation of dense bodies [12,13]. Only recently, these cells were identified as pancreatic stellate cells, formerly named fat-storing cells, which show similarities in their retinoic metabolism and morphology to hepatic stellate cells and are considered as key players in the fibrogenesis in different pancreatic diseases [14-16]. Activation of pancreatic stellate cells is orchestrated by cytokines and components of the ECM as well [21,22]. Among the cytokines involved in this process, transforming growth factor beta (TGF β) is of particular relevance [17].

Figure 1. **Transformation of pancreatic stellate cells is characterized by cellular enlargement, loss of retinoid droplets and the expression of smooth-muscle alpha actin. Initiating stimuli include TGF β and TNF α from platelets, macrophages or acinar cells. The activated pancreatic stellate cell is sensitive to proliferative and fibrogenic cytokines leading to enhanced proliferation and extracellular matrix synthesis. Modulation of extracellular matrix degradation through MMP and TIMP secretion is also a feature of the active pancreatic stellate cell. TGF β, transforming growth factor beta; PDGF, platelet derived growth factor; TNF α, tumor necrosis factor alpha; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; ECM, extracellular matrix**



Pancreatic stellate cells in fibrogenesis of the pancreas

Cells producing extracellular matrix in the pancreas were described as "fibroblast-like" cells showing characteristics of myofibroblasts, e.g. expression of α-smooth muscle actin, synthesis of collagens and fibronectin, and the formation of dense bodies (microfilaments) [16,18]. The presence of retinoid containing fat-storing cells in the pancreas of mice, rats, and humans was already demonstrated in 1982 [19]. While a potential role of these cells in pancreas remodelling and fibrosis was already discussed by Ikejiri et al. [20], it took another 8 years until Bachem and coworkers were able to isolate and characterize these cells [14]. Their data on description of this cell type have been supported in the same year by Apte and coworkers [15]. Because of their similarity in morphology and retinoid metabolism to hepatic stellate cells, Bachem et al., named these cells pancreatic stellate cells (PSC). PSC's are located in the interlobular and interacinar region of the human pancreas with characteristics of myofibroblasts, e.g. expression of α-smooth muscle actin (α-sm-actin), the formation of dense bodies (microfilaments) and the presence of retinoid-containing fat droplets [14,16] (*Fig. 1*). During primary culture of PSC, however, the number and size of retinoid fat droplets decrease in parallel to the increase in α-sm-actin expression and extracellular matrix synthesis. This switch from a quiescent "fat-storing" to a highly proliferative "myofibroblast-like" phenotype is associated with loss of cellular retinol content, the development of a prominent endoplasmatic reticulum, and increased synthesis of extracellular matrix proteins. In contrast to the "fat-storing" cell-type, the "synthetic" phenotype of PSC synthesizes and secretes high amounts of collagen type I and fibronectin [14]. By quantitative measurement of the procollagen peptides, we have demonstrated that the "synthetic" phenotype of human PSC synthesizes 25-40 fold more collagen type I than collagen type III esters [14]. Together, these studies identified the pancreatic stellate cells as a major source of extracellular matrix proteins.

Fibrogenesis in acute pancreatitis

In acute pancreatitis significant amounts of extracellular matrix are deposited in interlobular and interacinar regions of the pancreas. This has been extensively studied in the model of cerulein pancreatitis [23-25]. Pancreatic regeneration from acute cerulein-induced pancreatitis in rats is characterized by proliferation of acinar and centroacinar cells, an increase in mitotic activity of fibroblasts and by stimulation of transcription, synthesis and deposition of extracellular matrix components, in particular collagens I/III and fibronectin [23-25]. However, approximately two weeks after induction of pancreatitis the histology, organ weight and collagen content of the pancreas returned to control values indicating complete regeneration

[23]. Interestingly, even repetitive induction of cerulein pancreatitis failed to induce fibrosis in the rat pancreas [24]. Thus, the cerulein pancreatitis as a model of acute pancreatitis is characterized by a dynamic process of ECM-formation and removal, making it ideally suited for functional studies of the ECM in the pancreas.

Fibrogenesis in chronic pancreatitis

In chronic pancreatitis fibrosis is the most impressive morphological finding. The current model of molecular pathogenesis of fibrosis demonstrates the central role of activated pancreatic stellate cells. Chronic pancreatitis is characterized by destruction of acinar cells and islet cells, activation of pancreatic stellate cells and replacement by connective tissue. This connective tissue results from an increased deposition and disorganization of extracellular matrix proteins including fibronectin, laminin, and collagens type I, III and IV [26]. We and others could show that these alterations of ECM-proteins are accompanied by an increase of the transcript levels of genes coding for collagens I/III and IV, fibronectin and laminin in human chronic pancreatitis [27]. Ongoing studies are focusing on the identification and characterization of signaling regulated transcription factors, such as the Smads and Sp1 that might play important roles in pancreatic stellate cell activation and expression of other extracellular matrix components [29].

Fibrogenesis in pancreatic cancer

Pancreatic cancer tissues shows a strong desmoplastic reaction, characterized by a remarkable increase of interstitial connective tissue [28,29]. This desmoplastic reaction in pancreatic cancer tissue is accompanied by increased steady state levels of the mRNA's for collagens type I, III and IV, fibronectin and laminin [28] and of collagen protein. Connective tissue cells in the stroma of pancreatic carcinomas were shown to be the main site of collagen type I and III mRNA transcription by in-situ hybridization. Interestingly it appeared that connective tissue cells neighbouring the tumor cells contained larger amounts of collagen transcripts than stromal cells distant from the tumor, suggesting that stromal cells appear to be the main site of ECM production in pancreatic cancer [30].

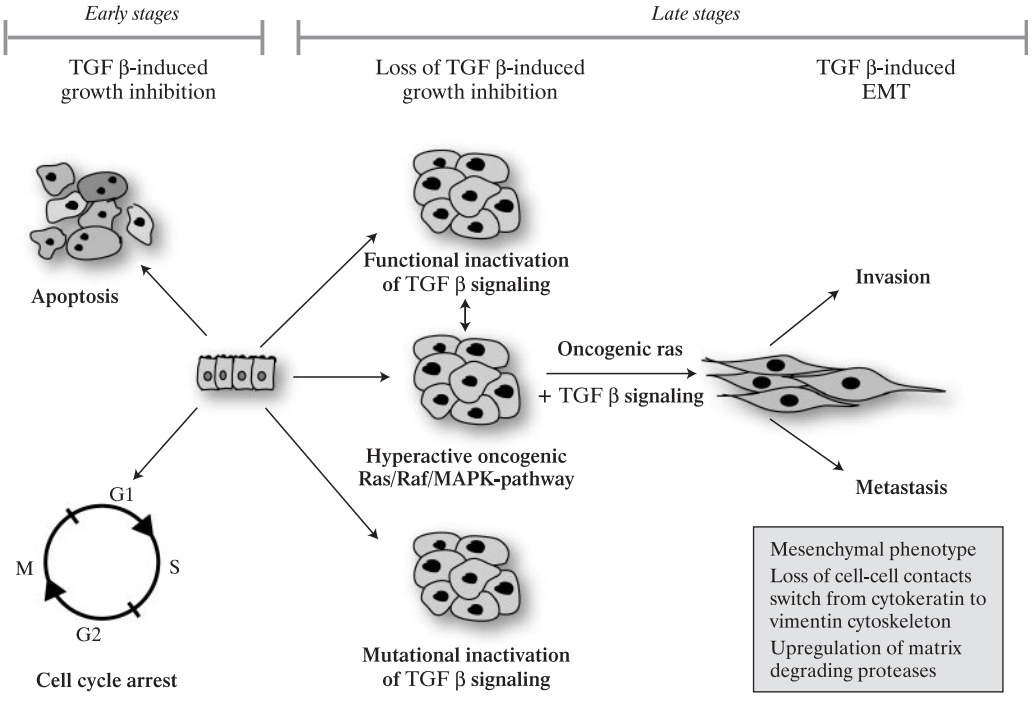
The role of TGF β and other growth factors in the regulation of pancreatic fibrogenesis

TGF β has been shown to be of particular importance in biosynthesis and turnover of extracellular matrix [31,32]. TGF β1 stimulates transcription and biosynthesis of different extracellular matrix proteins and in addition functions as an inhibitor of epithelial cell proliferation [33-35]. We could show that during regeneration from cerulein-induced pancreatitis in rats, alterations of collagen gene expression were accompanied by parallel alterations of TGF β1 gene expression [36]. A maximal increase of TGF β-mRNA (3-fold over controls) was found two days after the end of maximal cerulein infusion, and in-situ hybridization showed that these transcripts were mainly produced by both, stromal cells and acinar cells. TGF β1 protein content in total pancreas reached peak values after one day and protein levels remained high throughout the second day.

Additonal experiments using neutralizing antibodies against TGF β1 demonstrated that inhibition of TGF β during regeneration from cerulein-induced pancreatitis is associated with decreased levels of both collagen type I and TGF β, suggesting that TGF β plays an important role in the regulation of extracellular matrix proteins during regeneration from acute pancreatitis. The latter effect has been attributed to an autocrine loop in which TGF β induces its own synthesis (35,36).

Further evidence for the role of TGF β in pancreatic fibrogenesis was provided by two studies using transgenic mice models overexpressing TGF β. TGF β was expressed in pancreatic cells under the control of the insulin promoter to study the effect of this growth factor on the pancreas in an in-vivo situation [35,37]. Both studies reported cellular infiltration comprising macrophages and neutrophils, fibroblast proliferation and abnormal deposition of extracellular matrix in the pancreas. Böttinger and collaborators [38] have used an elegant approach based on the expression of a dominant-negative mutant TGF β type II receptor in transgenic mice to functionally inactivate TGF β signalling in epithelial cells. The dominant-negative mutant type II TGF β receptor blocked signaling by all three TGF β isoforms in primary hepatocyte and pancreatic acinar cell cultures generated from the transgenic mice. Acinar cells in the pancreas of these transgenic mice showed increased proliferation and severely perturbed acinar differentiation. Additional abnormalities in the pancreas included fibrosis, neoangiogenesis and mild macrophage infiltration. Data obtained in the experimental models described above clearly identify TGF β as one of the central regulators of ECM-formation in the pancreas. Moreover it becames clear, that TGF β which is released from various cell types including activated macrophages and platelets induces pancreatic fibrogenesis mainly through its effects on pancreatic stellate cells. It has been shown, for instance, that TGF β promotes PSC activation and increases expression of collagen and other ECM components through induction of an autocrine feedback mechanism [39]. The autocrine feedback loop is initiated through binding of TGF β to a heteromeric complexe of specific type II (TβR-II) and type I (TβR-I) kinase receptors. Receptor activation leads to phosphorylation of the Smad transcription factors, which then translocate to the nucleus and regulate the transcription of various target genes including TGF β itself, and genes involved in PSC activation and ECM synthesis. Interestingly, however, TGF β not only has the capacity to activate transcription of extracellular matrix forming proteins such as collagen I, collagen III and fibronectin but also induces the expression of matrix degrading proteinases such as MMP-2, MMP-9, and MMP-13 [40]. Although abundant data clearly demonstrate the critical role of the Smads in TGF β signaling and transcription, growing evidence suggest that TGF β can also induce the expression or activation of other transcription factors that do not belong to the Smad family but yet might play significant roles in TGF β-induced stellate cell

***Figure 2*. The dual role of TGF β in pancreatic cancer. In early tumor stages, TGF β acts as a tumor suppressor through transcriptional regulation of genes involved in cell cycle control or induction of apoptosis. In later stages of the disease, however, the tumor cells escape from TGF β-growth inhibition and respond to TGF β with increased invasion and metastasis**



activation. Though most of the data on the regulation of Smad signaling were obtained from studies on hepatic stellate cells, it appears most likely that Smad signaling activity in pancreatic stellate cells is also tightly controlled temporally and spatially through multiple mechanisms at the extracellular, membrane, cytoplasmic and nuclear levels. For example, Smad2 was found to be phosphorylated and activated in PSC's depending on the transdifferentiation grade and is negatively regulated through the inhibitory feedback loop imposed by Smad7. On the other hand, positive regulation of the Smads in PSC's occurs through activation of ERK MAP kinase signaling which appears to play a crucial role in Smad-mediated induction of the autocrine TGF β loop [41].

Taken together, the discovery of PSC's and its regulation through TGF β-induced signaling and transcription revealed new insights into the pathogenesis of pancreatic fibrosis and identified these highly active cells as the main cellular source of extracellular matrix in chronic pancreatitis. Thus, recent data propose a new model of the molecular pathogenesis of pancreatic fibrosis which suggests that acinar cell injury is followed by monocyte/macrophage invasion and activation, as well as platelet aggregation in areas of inflammation. This causes release of several polypeptide growth factors and in particular TGF β, which, in turn, stimulate PSC to synthesize extracellular matrix proteins in the pancreas.

This is also true for pancreatic cancer, in which TGF β has been demonstrated to play a key role in tumor cell invasion and in the development of the desmoplastic reaction. Based on studies using cultured cells, transgenic mice, and human tumors, an emerging model suggests a dual role for TGF β-signaling in tumorigenesis [42,43]. In early tumor stages, TGF β inhibits cell proliferation, at least in part, through transcriptional regulation of target genes involved in cell cycle control. During tumorigenesis, however, many tumor cells lose their ability to respond to TGF β with growth inhibition, and instead, respond with increased invasion and metastasis. Thus, loss of sensitivity to inhibition of growth by TGF β by most tumor cells is not synonymous with complete loss of TGF β signaling, but rather suggests that tumor cells gain advantage by selective inactivation of the tumor suppressor activities of TGF β while retaining its tumor promoting activities. We and others have shown that TGF β promotes tumor progression via stimulation of tumor cell invasion and metastasis, increased angiogenesis, and depression of local immune responses [44-46]. A hallmark of TGF β mediated tumor progression is the transdifferentiation of epithelial tumor cells into a fibroblast-like phenotype. We have shown that this so-called EMT (epithelial-mesenchymal transition) is strongly induced by TGF β, and is accompanied by upregulation of mesenchymal markers (e.g. collagen I and vimentin) and down-regulation of cytokeratins [46]. TGF β mediated EMT is further characterized by strong induction of uPA and MMP-2 expression and activation and results in increased motility, matrix degradation and tumor cell invasion [45]. Together, TGF β plays a multifunctional role in various pancreatic diseases ranging from modulator of tissue regeneration to stimulator of tumor cell progression.

The role of matrix metalloproteinases and their inhibitors in fibrogenesis

Matrix metalloproteinases (MMP) comprise a growing family of proteolytic enzymes that contain tightly bound zinc [45,46]. According to their substrate specificity MMP's can broadly be classified as collagenases, gelatinases, stromelysins and the membrane-type metalloproteinases. MMP's are secreted as proenzymes which are activated by proteolytic cleavage of an aminoterminal propeptide e.g. by plasmin, cathepsin G, trypsin, α-chymotrypsin and MMP-3 [47,49]. Activity is further controlled by various proteinase inhibitors such as α2-microglobulin and more importantly the family of tissue inhibitors of metalloproteinases, TIMP-1-4. Active MMP's display proteolytic activity for at least one component of the ECM. There is considerable evidence that MMP's have a major role in physiological ECM resorption as in development or postnatal remodeling and in pathological ECM-resorption e.g. associated with local invasiveness or metastasis of malignant tumors and the destruction of joints in rheumatoid arthritis [45-49].

Alterations of the balance of expression between MMPs and TIMPs has been described in several inflammatory diseases. In chronic pancreatitis, increased levels of MMP-2 (72 kDa collagenase IV), TIMP-1 and TIMP-2 have been detected, although the magnitude of MMP/TIMP transcript levels do not correlate to the degree of fibrosis and inflammation [50,51]. Interestingly, transcripts of genes encoding extracellular matrix degrading proteinases with a substrate specificity for interstitial extracellular matrix components such as MMP-1 and MMP-3 are not elevated in chronic pancreatitis. Possibly, the lack of MMP-1 and MMP-3 expression contributes to the deposition of ECM components in the interstitial space.

For a more functional approach to the role of MMP's and their inhibitors in the development of pancreatic fibrosis we used the model of cerulein-induced pancreatitis in rats [52]. Surprisingly increased expression during days 2-4 after induction of pancreatitis could only be demonstrated for MMP-2 and MMP-3, whereas transcript levels of MMP-1 and MMP-9 did not change throughout the regeneration period.

The role of MMPs and TIMPs has been extensively studied in tumorous diseases. Mostly, expression of MMPs and TIMPs was shown to correlate with an increased metastatic and invasive potential of tumor cells [53-55]. High expression and activation levels of MMP-2 have been found in various human cancer tissues, including breast and pancreatic cancer and correlated with tumor stage and grade in several cases [56,57]. We have recently demonstrated that increased expression and activation levels of MMP-2 are strongly associated with elevated expression levels of its activators MT1-MMP and MT2-MMP and that it plays a significant role in TGF β-mediated pancreatic tumor cell invasion [51]. A good correlation was also seen between MMP-2 expression and levels of transcripts coding for extracellular matrix proteins, the amount of collagen protein and the severity of the desmoplastic reaction. In-situ hybridization studies localized transcripts coding for MMP-2 in both, stromal and tumor cells, although it appeared to be more abundant in stromal cells.

Besides the family of metalloproteinases the plasminogen activator/plasmin system is considered as a key player in tumor invasion/metastasis. Tissue-type plasminogen activator (tPA) [58], urokinase-plasminogen activator (uPA) and the uPA-receptor (uPAR) [59] were found to be overexpressed in a variety of epithelial tumor tissues including pancreatic cancer in correlation with decreased postoperative survival [59]. Recently we identified a novel, membrane bound Kunitz-type serine proteinase inhibitor highly overexpressed in pancreatic cancer, which was named "kop" (for Kunitz-type proteinase inhibitor overexpressed in pancreatic cancer) [60]. The same proteinase inhibitor was cloned in human placenta and was called "bikunin". Placenta as pancreatic cancer is a tissue characterized by extensive ECM remodelling requiring the balanced activity of proteinases and proteinase inhibitors [61]. Recently published data suggest that kop/ /bikunin is a strong inhibitor of human plasmin, human tissue kallikrein and human plasma kallikrein [62] and might therefore be involved in the regulation of MMP-activation or directly counteract uPA.

Conclusions

Deposition of extracellular matrix in inflammatory and tumorous diseases of the pancreas is the result of a dynamic process of mechanisms like acinar cell injury and necrosis, inflammation, activation of macrophages, aggregation of platelets, release of growth factors, activation of pancreatic stellate cells, and stimulated synthesis of extracellular matrix and reduced matrix degradation. Pancreatic stellate cells represent the main cellular source of extracellular matrix in chronic pancreatitis and pancreatic cancer. Pancreatic stellate cells share homologies with hepatic stellate cells including storage of retinyl palmitate, retinal esterification, expression of the cytofilaments vimentin and desmin and phenotypic transition to an active matrix producing myofibroblast-like cell.

Among other growth factors, TGF β has been shown to be of paramount importance for the regulation of the dynamic process of extracellular matrix turnover in the pancreas. Proteinases as e.g. metalloproteinases or serine proteinases are TGF β-regulated effector proteins that are responsible for the coordinated removal of ECM components. Imbalances of the ratio between proteinases and their natural inhibitors may be one the major pathogenetic factors leading to fibrosis.

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Surgical treatment of constipation

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Abstract

Constipation is a common symptom in clinical practice. Definition of constipation includes abnormal bowel frequency, difficulty during defecation and abnormal stool consistency. There are many classifications of constipation based on constipation etiology (constipation in healthy people caused by life style, constipation as a symptom of digestive tract diseases, secondary constipation in the course of systemic disorders or associated with drugs) and/or constipation mechanisms (functional, mechanical). The numerous disorders leading to constipation make often diagnostic management difficult and complicated. Treatment of constipation includes dietary and behavioral approaches, pharmacologic therapy and in selected patient surgical treatment. Surgical treatment is recommended in young patients with severe slow transit constipation refractory to conservative treatment. Confirmation of indication to surgical treatment requires studies of colonic and anorectal function (colonic transit studies, anorectal manometry, studies of defecation). Preferred surgical technique is colectomy with ileorectal anastomosis. Authors reported good results and patient satisfaction in 50-100 percent of cases. Postoperative complications include intestinal obstruction, abdominal pain, flatulence, diarrhea.

Key words: constipation, surgical treatment.

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Definition

Diagnostic criteria for constipation include:
- abnormal stool form (at least 25 percent lumpy or hard stools),
- abnormal stool passage (at least 25 percent defecations with straining and feeling of incomplete evacuation, manual maneuvers to facilitate more than 25 percent defecations) and/or
- abnormal stool frequency (less than 3 bowel movements per week) [1].

Severe constipation is diagnosed when there are 4 or less bowel movements per month.

Epidemiology

Constipation is one of the most common complaints in Western countries. Epidemiological studies in North America reported frequency of constipation from 1.9 to 27.2 percent (most from 12 to 19 percent) of adults. Approximately 63 million people in North America suffer from this symptom. There is females predominance among constipated patients (female to male ratio – 2.2 : 1). The prevalence of constipation increases with age [2]. In the study of 10 000 adults in the USA prevalence of constipation was 14.7 percent with 45 percent of individuals having the symptom for 5 years or more [3].

Etiology

As there is a great number of disorders causing constipation there are also numerous classifications of constipation. They usually based on constipation etiology or constipation mechanism. Different criteria are used to precise categorization of constipation, but it is still difficult to find one classification including all constipation types. For example Hirschprung's disease may be categorize to congenital disorders and simultaneously to the group of constipation secondary to peripheral neurogenic disorders as well as to the group of constipation with large bowel dilatation.

Table 1. **Classification of constipation (according to Rocha Miranda JA, Wexner SD: Surgical treatment of constipation [in]: Shackelford's surgery of the alimentary tract. Zuidema GD, Yeo CJ. W.B. Saunders Company, Philadelphia 2002, 431-445** [9]

Congenital	**Extraintestinal**	Metabolic and endocrine
Hirschprung's disease	Pharmacologic	
Acquired	Analgesics	Amyloidosis
Chagas' disease	Anesthetics	Diabetes
Mechanical	Anticholinergics	Hypercalcemia
Obstructive	Anticonvulsant	Hyperparathyroidism
Neoplasia	Antidepressants	Hypokalemia
Adhesions	Antiparkinsonian agents	Hypopituitarism
Hernia	Antacids	Hypothyroidism
Volvulus	Barium sulfate	Pheochromocytoma
Endometriosis	Diuretics	Porphyria
Severe sigmoid diverticulitis	Ganglionic blockers	Pregnancy
Anal stenosis	Iron	Scleroderma
	Hypotensives	Uremia
Functional	Laxative abuse	
Inadequate fiber intake	Metallic intoxication	**Neurogenic**
Irritable bowel syndrome	(arsenic, lead, phosphorus)	
	Monoamine oxidase inhibitors	Peripheral
Idiopathic	Opiates	Autonomic neuropathy
	Paralytic agents	von Recklinhausen's disease
Colonic	Parasympatholytics	Multiple endocrine neoplasia IIb
Inertia	Phenothiazines	
Dolichocolon	Psychotherapeutics	Spinal
Pelvic		Cauda equina tumor
Intussusception/rectal prolapse		Iatrogenic
Rectocele		Meningocele
Sigmoidocele		Multiple sclerosis
Descending perineum		Paraplegia
Paradoxical puborectalis contraction		Resection of nervi cringens
Perineal hernia		Shy-Drager syndrome
		Tabes dorsalis
		Trauma
		Central
		Parkinson's disease
		Stroke
		Tumors

Constipation in healthy people

In healthy people constipation occurs occasionally as a result of diet, traveling or emotional factors. Chronic constipation may be caused by: low – fiber diet (recommended amount of dietary fiber is 20-35 g per day), intake of products increasing tendency to constipation (tea, cacao), low consumption of fluids, low level of physical activity. All of this factors led to impairment of large intestine transit.

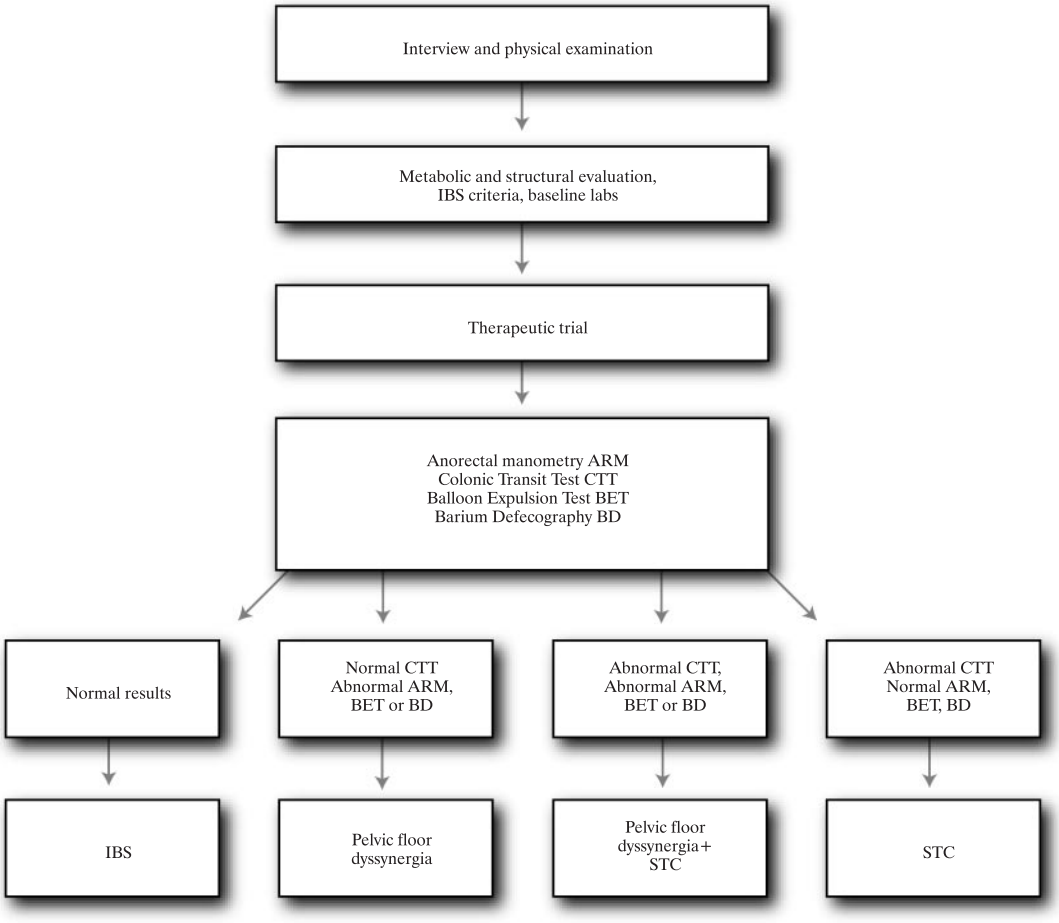
Constipation secondary to the systemic diseases and drugs

Constipation is associated with numerous disorders affecting colonic motility: metabolic and endocrine disorders (the most common are diabetes mellitus and hypothyroidism), neurogenic disorders (peripheral, central, spinal), muscle disorders. Neurogenic, peripheral disorders include congenital (Hirschprung's disease) or acquired (Chagas' disease) disorders of the enteric nervous system. It must be remembered that every chronic disease may be a cause of constipation because of impairment of physical activity, especially in bedridden patients, change in diet and concomitant medications. Drugs which can produce or exaggerate constipation are shown in *Tab. 1*.

Constipation in the course of organic and functional digestive tract diseases

Constipation is usually a symptom of digestive tract disorders. Organic disorders cause constipation in mechanical way (obstruction). Mechanical impairment in colonic transit may be caused by: colorectal cancer, diverticular disease, intraperitoneal adhesions etc. Functional bowel disorders are diagnosed basing on Rome II criteria and excluding other condition with similar clinical presentation. According to Rome II criteria constipation is a symptom of irritable bowel syndrome (IBS) and functional constipation[1]. Colorectal motility disorders presenting with constipation include: slow-transit constipation (STC, colonic inertia), pelvic floor dysfunction and combination syndromes. STC typically affects young women, with the onset of symptoms below age 25 in most cases. Etiopathogenesis of

Figure 1. **Diagnostic algorithm for refractory constipation [14,15]**



STS is not clear. Wedel et al. [4] reported that the colonic motor dysfunction in STC is associated with quantitative alterations of the enteric nervous system (oligoneuronal hypoganglionosis), which can not be detected by submucosal biopsy because they primarily affect myenteric plexus and external submucous plexus. Pelvic floor dyssynergia (anismus) is described as inappropriate contraction or failure to relax the pelvic floor during the attempt to defecate. Behavioral treatment of this disorder (biofeedback) is reported to be successful in 80 percent of cases [5]. Idiopathic megarectum and megacolon are defined as dilatation of the rectum and/or colon without demonstrable organic disease [6,7]. Other condition with large bowel dilatation and constipation is intestinal pseudo-obstruction. Mann et al. [8] found out that constipation occurred in 40 percent of patients with intestinal pseudo-obstruction. Classification of constipation is shown in *Tab. 1*.

Diagnostic strategies

In the evaluation of the patient presenting with constipation history of complaint and physical examination are mandatory. The aim of the other studies is exclusion of anatomic (colonoscopy, barium enema) and extracolonic causes of constipation. The next step is 6-month period of conservative approach: dietary modification, physical exercises, behavioral and pharmacologic treatment. After failure of such management referral for colonic and anorectal physiologic testing should be considered (*Fig. 1*).

Colonic and anorectal testing techniques

Evaluation of colonic and anorectal function includes several tests: colonic transit time, anorectal manometry, defecography, EMG and pudendal nerve terminal latency (PNTML), balloon proctography, perineometry, scintigraphic study of rectal evacuation. Results of colorectal function tests allow to categorize the constipation to 4 groups: 1. colonic inertia with/without megacolon, with/without gut dysmotility syndrome, 2. pelvic floor dysfunction with anatomical abnormalities (Hirschprung's disease, perineal descent, rectocele, sigmoidocele, intussusception, rectal prolapse) or without anatomical abnormalities (paradoxical puborectal contraction, levator spasm, anismus, rectal pain), 3. combined syndrome, 4. normal transit constipation [10].

Colonic transit study involves ingestion of radiopaque markers and measurement of their transit by abdominal

radiographs. Overall colonic transit time and segmental transit times can be calculated. Study is conducted until at least 80% of markers have passed or during a defined period of time (6-8 days). In the Metcalf's method markers are ingested on three consecutive days and on day 4 and day 7 their distribution is assessed by an abdominal plain film [11]. To determine localization of the markers in the left, right and rectosigmoid colon bony landmarks are used. Colonic transit time can be also determined by the radioisotopes. This test confirms patients subjective complaint of constipation and/or decreased bowel frequency and is useful for confirmation of slow transit and identification of the colonic regions with delays in transit.

Defecography (evacuation proctography) is radiological contrast study evaluating process of defecation using fluoroscopic techniques. This method is helpful in patients with suspected pelvic floor dyssynergia (inappropriate contraction of the puborectalis muscle), enterocele and anterior rectocele. Defecography supports the symptom of inability to defecate.

Anorectal manometry provides information about anal canal pressure and anal sphincter responses. Procedure includes a number of specific tests: resting anal pressure, anal canal squeeze pressure, rectoanal inhibitory reflex, anal pressure in response to a cough, anal canal pressure in response to defecatory maneuvers, compliance of the rectum in response to balloon distension, sensory thresholds in response to balloon distension. Anal manometry confirms the diagnosis of pelvic floor dysfunction and is also useful in diagnosis of Hirschprung's disease. Authors emphasize that clinical value of anorectal manometric tests is limited by the relative absence of standardization of test protocols and normative data from a large number of healthy individuals [12]. Balloon expulsion test estimates motor function and coordination. This procedure confirms the symptom of inability to defecate. Balloon distension test evaluates rectal sensation detecting the threshold for: rectal sensory (the first detectable sensation), the sensation of urgency to defecate and the sensation of pain (maximum tolerable volume). The procedure is used in differential diagnosis between functional and neurological causes of constipation [13].

Treatment of constipation

Treatment of constipation includes:

- life style modification (adequate intake of dietary fiber and fluids, regular physical activity),
- behavioral approaches (habit training, biofeedback),
- pharmacologic treatment (bulk-forming laxatives, emollient laxatives, hyperosmolar laxatives, salina laxatives, stimulant laxatives, antraquinones),
- surgical treatment [16].

Surgical treatment of slow-transit constipation

Surgical treatment is indicated in selected patients with constipation. Excluding patients with constipation as a symptom of organic disease of digestive tract in which surgery is a treatment of choice (colorectal cancer, complicated diverticular disease etc.) surgery is used in Hirschprung's disease, slow-transit constipation, rectocele, enterocele, rectal intussusception and prolapse.

Surgery as a method of treatment for refractory constipation was first described by Sir William Arbuthnot-Lane in 1908. He reported 20 percent mortality rate and 64 percent success rate in the group of 39 patients.

Currently, the most widely accepted operative technique for STC is colectomy with an ileorectal anastomosis. Other procedures used in surgical treatment of STC involve: colectomy with ileosigmoid anastomosis, segmental resection of the colon with colo-colonic or colorectal anastomosis.

According to Lahr et al. [17] operative treatment of constipation may be consider when this symptom meets several criteria, including: "1) the severity must warrant the surgical risks, 2) medical and psychological causes have been ruled out, 3) medical therapy has failed, and 4) diagnostic studies show correctable anatomic or physiologic abnormalities". Lahr et al. reported outcome of operative treatment for constipation in the group of 196 patients. 44 percent of patients underwent pelvic hiatal hernia repair (sigmoid resection, rectopexy and patch sacral colpopexy), 27 percent underwent total abdominal colectomy and ileorectal anastomosis for colonic inertia and 29 percent had surgery for both colonic inertia and pelvic hiatal hernia. Relief of symptoms was reported by 90 percent of patients. Complications occurred in 6.1 percent of patients and included: small bowel obstruction, anastomotic leak, ureteral stenosis, patch erosion.

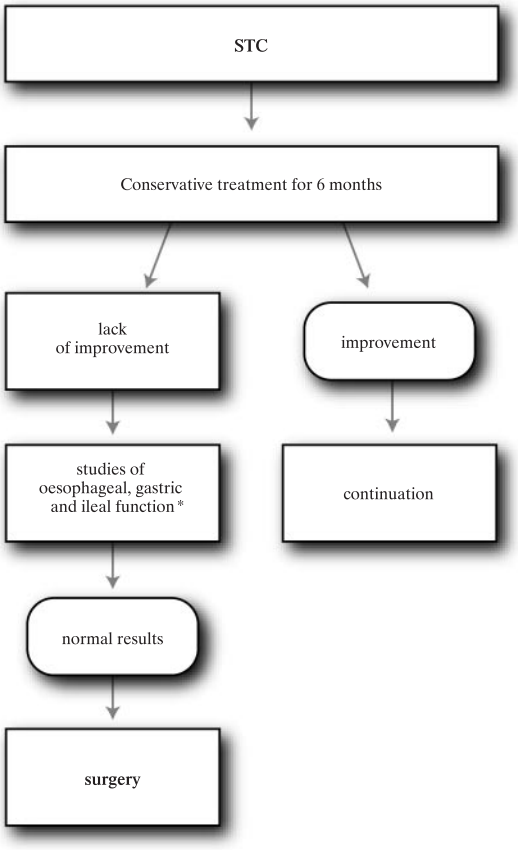
Upper gastrointestinal motility disorders are often recognized in patients with STC and may be an important predictive factor for postoperative morbidity (intestinal obstruction). Patients with oesophageal, gastric and small-bowel motor abnormalities have 50 percent chance of recurrent symptoms or other abdominal complaints after operation [18].

Piccirillo et al. [19] presented the results of prospective assessment of total abdominal colectomy and ileorectal anastomosis in the group of 54 patients with STC. 94 percent of patients had satisfactory improvement in bowel habit with a mean frequency of spontaneous bowel movements of 3.7 per day (range 1-10) after a mean follow-up of 27 months. Small bowel obstruction appeared in 9 percent of patients. Authors suggested that high success rate in the study is probably due to the strict patient selection with exclusion of paradoxical puborectalis muscle contraction and Hirschprung's disease.

Verne et al. [20] also emphasizes the importance of preoperative patient selection with careful evaluation of gastrointestinal motility. They found out that patients with abnormal 24-h intraduodenal manometric findings reported higher mean postoperative pain scores, had longer hospitalization and higher rate of readmission to the hospital during the follow-up comparing to the patients with normal upper gastrointestinal motility. Authors concluded that subtotal colectomy has a long term benefit in patients with colonic inertia (improvement in abdominal pain and frequency of the bowel movements). They also confirmed better results in patients with colonic inertia without upper gastrointestinal dysmotility.

Glia et al. [21] studied outcome of colectomy for STC in patients with normal and abnormal antroduodenal manometry findings. Studied group consisted of 17 patients (median age 46 years) suffering from STC, who underwent subtotal colectomy.

Figure 2. **Algorithm for STC**



* oesophageal manometry, antroduodenal manometry, scintigraphic study of gastric emptying, lactulose breath hydrogen test to measure orocecal transit, scintigraphic study of small bowel transit

Preoperative evaluation included whole gut transit time, anorectal manometry, antroduodenal manometry, EMG of the anal sphincter, balloon expulsion test and defecography. 56 percent of patients had abnormal antroduodenal manometry. Authors reported that overall outcome of colectomy was good or excellent in 71 percent of patients with a trend toward better outcomes in patients with normal antroduodenal manometry. At long-term follow-up (5 years) 86 percent of patients reported improvement after operative treatment despite persisted pain (43%) and bloating (50%).

Fitz Harris et al. [22] studied the relationship between functional outcomes and quality of life after subtotal colectomy for STC in the group of 75 patients. All patients were female. Authors used the Gastrointestinal Quality of Life Index (GIQLI) designed to evaluate gastrointestinal symptoms, physical, psychological, social and disease-specific issues. The maximum possible score in GIQLI is 144 (zero /worst/ and four /best/ scores for each question). 77% patients had low sigmoid anastomoses and 23 percent had rectal anastomoses. Assessing functional outcome and complications authors found out that: 5 percent of patients in studied group reported recurrent or persistent constipation, 41 percent persistent abdominal pain, 69 percent diarrhea, 45 percent incontinence. Four percent of patients required a permanent ileostomy and 17 percent of patients underwent lysis of adhesions. Assessing the quality of life authors found out that the mean GIQLI score was 103 ± 22. Abdominal pain, diarrhea and incontinence were recognized as the symptoms with the strongest impact on GIQLI score. 93 percent of patients stated they would undergo subtotal colectomy again if given a second chance. Authors conclude that defecation frequency increased in 95 percent of patients after subtotal colectomy but a significant number of patients suffer from gastrointestinal symptoms which well correlate with the GIQLI score.

Kamm et al. [23] reported long-term efficacy of surgical treatment for constipation of 50 percent. 1/3 of patients suffered from diarrhea and 10 percent from recurrent constipation.

Bielecki and Kamiński [24] presented outcome of surgical treatment (colectomy with ileorectal anastomosis, subtotal colectomy with anastomosis of small stump of caecum with rectum) in the group of 10 patients with severe slow-transit constipation. Postoperative complications occurred in 3 patients (deep vein thrombosis, pneumonia, subileus). After a follow-up period (6-58 months) all patients were satisfied with operative treatment although most of them suffered from sporadic abdominal pain and bloating.

Some authors present laparoscopically assisted subtotal colectomy for STC as a possible alternative method to the open procedure, however the data are limited [25].

Surgery is also offered as a method of treatment of severe functional constipation in children [26] (*Fig. 2*).

Conclusions

In selected patients with severe constipation surgery appears to be a valuable method of treatment. Effectiveness of surgical intervention and prevalence of postoperative complications are determined by very careful preoperative assessment of gastrointestinal function. There is a great number of gastrointestinal transit studies and anorectal testing techniques to confirm the slow-transit constipation and exclude gastrointestinal dysmotility syndrome. Before referral for surgical treatment all available methods of conservative treatment have to be used. Currently the most accepted procedure for STC is colectomy with ileorectal anastomosis with success rate of 50-100 percent. It has to be emphasized that symptoms coexisting with constipation (abdominal pain, bloating, flatulence) usually persist after operation. Selection of appropriate patients for surgical treatment through extensive studies is a condition of the satisfactory outcome.

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Duodenum preserving pancreatic head resection in the treatment of chronic pancreatitis

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Abstract

Chronic pancreatitis is an inflammatory disease which is characterized by a progressive conversion of pancreatic parenchyma into fibrous tissue. Most frequent causes are alcohol over-consumption, beside anatomic variants such as pancreas divisum, cholelithiasis or individual genetic predisposition. The process of fibrotic transformation with consecutive loss of pancreatic parenchyma leads to exocrine insufficiency and maldigestion, and in advanced stage of the disease to diabetes mellitus. In addition to exocrine and endocrine malfunction, mechanical complications such as formation of pancreatic pseudocysts, duodenal and common bile duct obstruction occur.

About 50% of the patients with chronic pancreatitis will need surgical intervention due to intractable chronic pain. Recent investigations suggest that the head of the pancreas triggers the chronic inflammatory process. Therefore, resection of this inflammatory mass must be regarded as the pivotal part of any surgical intervention. Radical techniques such as Whipple-procedure are undoubtedly successful regarding pain reduction. However, even in its pylorus preserving variant this technique is associated with a high postoperative morbidity due to large loss of pancreatic parenchyma and the loss of the duodenal passage.

30 years ago, H.G. Beger described for the first time the technique of duodenum preserving pancreatic head resection that better combines resection of the pancreatic head with low morbidity. Over the years different variations of the original Beger technique (Frey, Izbicky, Berne modification) have been developed, and the excellent results obtained with these techniques underline, that organ sparing procedures should be preferred in the surgical treatment of chronic pancreatitis.

Key words: chronic pancreatitis, chronic pain syndrome, DPPHR, diabetes mellitus, maldigestion.

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Introduction

Chronic pancreatitis is an inflammatory disease which is characterized by an irreversible conversion of pancreatic parenchyma to fibrous tissue. The incidence in the western world is up to 10/100 000 pa with rising morbidity of female [1]. Alcohol overconsumption accounts for most of the cases (75-90%), other reasons are idiopathic disease, anatomic variants such as pancreas divisum, cholecystolithiasis or genetic predisposition [2-4]. The fibrotic transformation of the pancreas with consecutive loss of intact parenchyma leads to exocrine insufficiency, maldigestion and weight loss, later on to endocrine insufficiency and diabetes mellitus. Beside endocrine and exocrine insufficiency, mechanical problems arise such as formation of pancreatic pseudocysts, duodenal obstruction and stenosis of the ductus hepatocholedochus [5] (*Fig. 1*).

Chronic pain syndrome

Abdominal pain is the leading symptom of chronic pancreatitis. 50% of the patients will need surgical intervention due to untractable pain during their lifetime [6]. The ethiopathogenesis of the chronic pain syndrome in chronic pancreatitis is not fully clarified [7]. Ductal hypertension due to protein plugs and stenosis, or intestinal compartment with local ischemia are traditionally believed to play a crucial role in

the pathogenesis of this disease. According to this hypothesis, reduced secretion of pancreatic enzymes should lead to a reduction of pain in chronic pancreatitis patients. However, this could not be proven clinically. Neither administration of pancreatic enzymes [8] nor octreotid [9,10] could influence frequency and intensity of pain attacks. Furthermore it is well described that even a burn out of the gland with complete loss of exocrine secretion does not lead to a significant pain release in chronic pancreatitis patients [11]. The hypothesis that different mechanisms have to be involved in the pathogenesis of pain generation in chronic pancreatitis is additionally supported by the observation, that surgical drainage procedures, even in case of a dilated pancreatic duct lead to pain relief only in about 50% of the patients [12].

Chronic pain syndrome and neuroimmune interaction

A recent pathophysiological concept interprets the generation of pain as an interplay between the nerve- and immune system [13-16]. Immunohistochemical analysis shows a high density of enlarged nerve fibres in chronic pancreatitis tissue [17]. Keith et al. could show that the pain level in patients with chronic pancreatitis correlated more with the degree of eosinophil infiltration of these enlarged nerves rather than with the degree of duct dilatation [18]. Electrone microscope analysis of these nerves reveals damaged perineurium and infiltration of leucocytes which may explain how pancreatic enzymes and mediators of inflammation enter neural structures and alter their structure and functioning [19]. Immunhistochemical analysis of chronic pancreatitis tissue revealed an altered pattern of intrinsic and extrinsic innervation with overexpression of different neurotransmitters such as “Substance P” und “Calcitonin Gene Related Peptide” (CGRP) in enlarged intrapancreatic nerves [20]. Since both cytokines are important pain transmitters, these findings provide evidence that alterations of pancreatic nerves themselves are involved in the pathogenesis of the disease and lead to the concept of neuroimmune interaction as a basic mechanism in the pathogenesis of CP and chronic pain syndrome.

This interesting hypothesis is confirmed by the fact, that the presence of growth-associated-protein-43 (GAP-43), an established marker of neuronal plasticity, correlates with individual pain scores in patients with CP [19].

Surgical therapy of chronic pancreatitis

Chronic pancreatitis is first of all a domain of conservative treatment. However, indication for surgical therapy is given when mechanical complications such as stenosis of the common bile duct or pancreatic duct, gastrointestinal obstruction due to the pancreatic head tumor or pseudocysts occur, or if untractable chronic pain leads to a significant reduction of the patient’s quality of life and ability. About 90% of the patients suffer from chronic abdominal pain, and in two third of the patients, untractable pain is the indication for surgical intervention.

In principle draining and resective surgical procedures have to be distinguished. The advantage of simple drainage procedures is, that no healthy tissue is sacrificed. However it is clear that drainage procedures do not remove inflamed tissue especially in the head of the pancreas that may be regarded as the pacemaker of the disease. For this reason simple drainage procedures are indicated only in well defined cases, and the absence of clear concept will result in unsatisfactory outcome of the individual patient [21,22].

Drainage procedures

On the base of ERCP and NMR-cholangio-pancreaticography findings, two different types of chronic pancreatitis can be distinguished: First the so-called “large duct“ – form which is characterized by a dilated pancreatic duct (>7 mm), and a “small duct” – form where the ductus wirsungianus is not dilated (4-7 mm) [23]. Distinguishing between these two forms has a certain impact on the operation technique. Drainage procedures are definitively not indicated in the small duct form of chronic pancreatitis where the entire gland is involved in the process of chronic inflammation and fibrosis. In contrast, in case of the large duct form, disturbed drainage of the pancreatic duct due to stenosis in the pancreatic head leads to its proximal dilatation [12]. In these cases, it may be discussed whether augmented intraductal pressure plays a role in the pathogenesis of chronic pain and whether drainage procedures like longitudinal pancreatico-jejunostomy may be indicated [24]. This technique is simple to perform, is associated with a low complication rate and allows for reliable drainage of the pancreatic tail and body with minimal loss of pancreatic parenchyma [21,22]. However, although performed in selected cases, this operation leads to satisfactory pain relief in only half of the patients [12,25,26]. This gives evidence that dilation of the pancreatic duct reflects ductal obstruction, but drainage of these ducts is only a part of a therapeutical concept. The point is, that a longitudinal incision of the gland will not allow for the drainage of the pancreatic head. Even if the pancreatic head seems to be normal in its aspect and diameter, irreversible neuroinflammatory alterations may be present that act as a pacemaker of the disease [12] and propose the chronic pain syndrome [18,27].

Resecting procedures

Over the years, the Kausch-Whipple procedure represented the surgical standard in the treatment of patients with chronic pancreatitis and complicated disease, and it could be shown that this operation could be performed in specialized centers with a very low mortality and morbidity rate [28-30]. However, while the complete removing of the pancreatic head assures good results regarding pain relief, the loss of duodenum and pylorus is associated with a relatively high morbidity and reduction of quality of life [23,31].

Especially in the United States, the pylorus preserving variant of pancreatic head resection became popular and

Table 1. **Results of duodenum-preserving pancreatic head resection**

	Author and year of publication	n=	Morbidity	Mortality	Pain free/-release	Follow-up (years)
BEGER	**Beger et al.** (1984)	57	19/57	1.8%	Fully rehabilitated 87%	2
	Bloechle et al. [50] (1995)	25	-	0	QoL index from 28 to 85	1.5
	Eddes et al. [51] (1996)	15	30%	0	73% / 86%	3.1
	Büchler et al. [52] (1997)	298	28.5%	1%	- / 88 %	6
	Izbicki et al. [53] (1997)	38	32%	0	- / 89%	2.5
	Beger et al. [43] (1999)	504	-	0.8%	78.8% / -	14
	Witzigmann et al. [54] (2002)	35	-	0	QoL index from 30 to 72	2
FREY	**Keus et al.** [55] (2003)	36	-	2.8%	- / 60%	4.6
	Frey et al. [44] (1994)	50	22%	0%	74% / 87%	3.1
	Izbicki et al. [56] (1995)	22	9%	0	- / 94%	1.5
	Izbicki et al. [23] (1998)	31	-	3.2%	- / 90%	2
	Kelemen et al. [57] (2002)	13	0	0	- / 57%	1.7
	Farkas et al. [58] (2003)	30	k.A.	0%	100%	0.8 years
BERN	**Farkas et al.** [59]	100	-	0%	92%	2.4 years
	Friess et al. *	42	0	14	81% / 93%	0.9 years

* unpublished data

represents more and more an alternative to the classical Kausch-Whipple procedure. Preservation of the pylorus and the first part of the duodenum allows for a controlled gastric emptying and reduces the incidence of dumping and gastric biliary reflux with consecutive gastritis. Regarding postoperative morbidity and quality of life parameters, the pylorus preserving pancreatico-duodenectomy is superior to the classical variant. 90% of the patients gain weight after the operation and 89-95% experience significant pain relief, although delayed gastric emptying can jeopardize the improvement of quality of life [32,33]. However, the principal disadvantage of pancreatico-duodenectomy in the treatment of patients with chronic pancreatitis remains. The loss of the duodenal passage has a negative impact on digestion and regulation of serum glucose level. In addition, about 45% of the patients will develop diabetes mellitus, due to the extended loss of pancreatic parenchyma [34,35]. This indicates that despite of relatively good results regarding pain reduction, this originally for the treatment of pancreatic malignomas designed intervention represents an over-treatment in this benign disease. Apart from single cases in which patients history or imaging cannot rule out malignancy, it is not justified, to sacrifice the duodenum and a part of the stomach and common bile duct to remove the inflammatory pancreatic head tumor.

Before CT and ERCP entered the scenery as diagnostic tools to identify an enlarged pancreatic head, left pancreatic resection was regarded as a standard procedure in the treatment of chronic pancreatitis with dilated pancreatic duct. Interestingly, the results obtained with this technique regarding pain release are not satisfactory. Only 55% of the patients experience an acceptable pain release after left pancreatic resection and the incidence of postoperative endocrine insufficiency is high due to the high density of islets in the tail of the gland [36]. Furthermore, satisfactory pain release after left pancreatic resection cannot be expected in even those cases, where CT and ERCP localize the inflammatory alterations in the tail of the gland. This important observation supports the hypothesis, that alterations in the pancreatic head are crucial for the progression of the disease and the development of the chronic pain syndrome. In conclusion, left pancreatic resection should be used only in single and well defined patients. We see the best indication for this technique in the treatment of isolated cysts of the pancreatic tail where chronic pain is not the indication for surgery [37,38].

The technique of "duodenum preserving pancreatic head resection"

Long before sophisticated imaging techniques were available, Hans Beger identified the pancreatic head as the pacemaker of chronic pancreatitis. In 1972, he was the first to describe a novel surgical technique which allowed the isolated resection of the pancreatic head without further organ loss [39-42]. If performed in specialized centers, the duodenum preserving pancreatic head resection can be performed with a very low morbidity and mortality [43,44]. The advantage of preservation of the duodenal passage is a nearly physiological regulation of enteral function and blood glucose level. In addition, preservation of islet rich parts of the pancreatic parenchyma in the tail of the pancreas results in a low incidence of postoperative diabetes mellitus compared to other resective procedures [37,39,40]. The effectiveness of this surgical technique on long-term pain release is high (>80% after a median follow-up of 5 years). Endocrine function is mostly impaired and a high rate of professional rehabilitation can be achieved (~70%) [7,39,40,43,45,46]. In all relevant aspects, duodenum preserving pancreatic head resection is comparable or even superior to more radical resective procedures (*Fig. 2* and *Fig. 3)*.

In 1985 Frey and Smith introduced a modification of duodenum preserving pancreatic head resection which combines a longitudinal pancreatico-jejunostomy with a local resection of the pancreatic head [47,48]. This technique combines the principle of duodenum preserving pancreatic head resection

Table 2. **Controlled randomized trials comparing different surgical approaches for the treatment of chronic pancreatitis**

Author and year of publication	Type of intervention	n =	Results
Klempa et al. [30] (1995)	Beger / ppWhipple	21 22	DEPKR / Whipple: shorter hospital stay (16d vs 21d, $p<0.05$), lower rate of exocrine insufficiency (4 vs 20, $p<0.05$), less analgetics (0 vs 6, $p<0.05$)
Büchler et al. [60] (1995)	Beger / ppWhipple	20 20	DEPKR / ppWhipple: pain free (75% vs 40%, $p<0.05$), better weight gain (4.1 vs 1.9, $p<0.05$), less frequent endocrine insufficiency ($p<0.01$)
Müller et al. [34] (1997)	Beger / ppWhipple	10 10	ppWhipple: delayed gastric emptiing ($p<0.05$), path. secretion pattern of enteral hormones ($p<0.05$)
Izbicki et al. [53] (1997)	Beger / Frey	38 36	DEPKR / Frey: comparable results regarding pain control (95% vs 94%), improvement of quality of life (both 67%), professional rehabilitation (74% vs 69%) and exocrine and endocrine function
Izbicki et al. [23] (1998)	Frey / ppWhipple	31 30	Frey: lower morbidity (19% vs 53%, $p<0.05$), improved quality of life (71% vs 43%, $p<0.05$), professional rehabilitation (68% vs 43%, $p<0.05$)

Figure 1. **Complications of chronic pancreatitis: stenosis of common bile duct – a, Wirsungian duct – b, duodenum – c and retropancreatic vessels – d. (Modified from [60])**

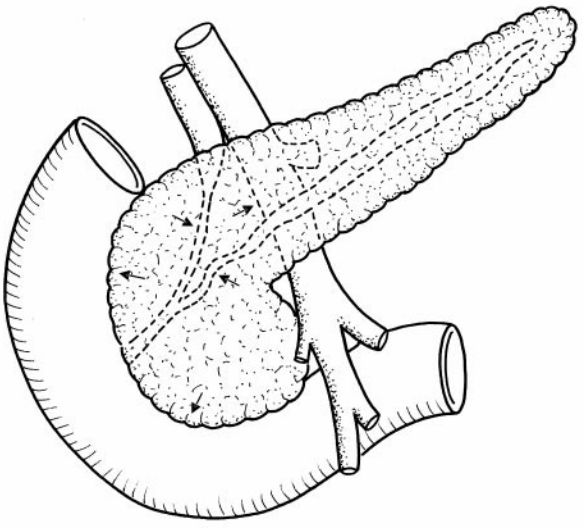
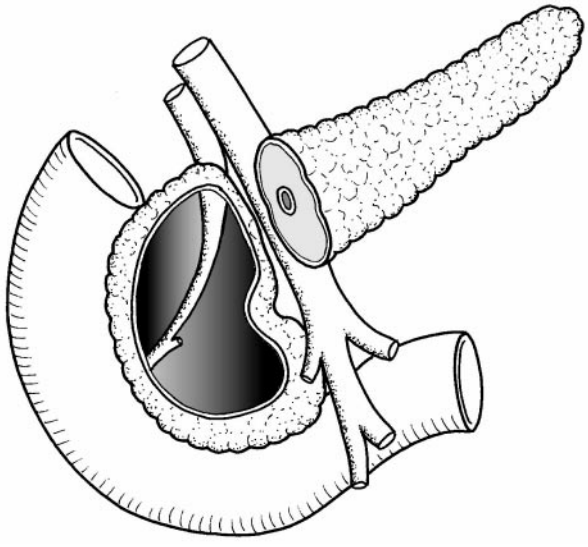


Figure 2. **Duodenum preserving pancreatic head resection according to Beger before reconstruction: decompression of common bile duct, Wirsungian duct, duodenum and retropancreatic vessels and division of the pancreatic body over the portal vein. (Modified from [60])**



with drainage of the ductus wirsungianus (*Fig. 4*). Compared to the original Beger procedure, this variant is simpler to perform as it spares the dissection of the pancreas from the portal vein and the division of the pancreatic body [23]. In a prospective randomized trial [23], both techniques were comparable regarding pain control (94% Frey vs 95% Beger), prevention of complications (91% Frey vs 92% Beger) and quality of life.

A similar approach to the surgical therapy of the small duct form of chronic pancreatitis is described by Izbicki [49]. He combines a duodenum preserving resection of the pancreatic head with a V-shaped longitudinal incision of the pancreatic body to also reach ductal side branches of II° und III° order. Although only a relatively small number of patient have been treated with this technique, results seem to be comparable to the original Beger technique. 30 patients were operated with a zero-mortality. Within a 30 months follow-up, 92% of the patients were pain free with preserved exocrine and endocrine pancreatic function. The median "Quality of Life" – index rose about 65% and professional rehabilitation was successful in 69% of the patients (*Tab. 1* and *Tab. 2*).

In order to combine the advantages of the well proven original Beger technique with the technique according to Frey, we developed another modification of duodenum preserving pancreatic head resection that combines the advantages of the two techniques and spares the technical demanding division of the gland over the portal vein in order to minimize the risk of intraoperative bleeding (*Fig. 5*). At the moment we are running a randomized trial in which we compare this technique with the

Figure 3. **Reconstruction after duodenum proserving pancreatic head resection according to Beger: end-to-side and side-to-side pancreatico-jejunostomy. (Modified from [60])**

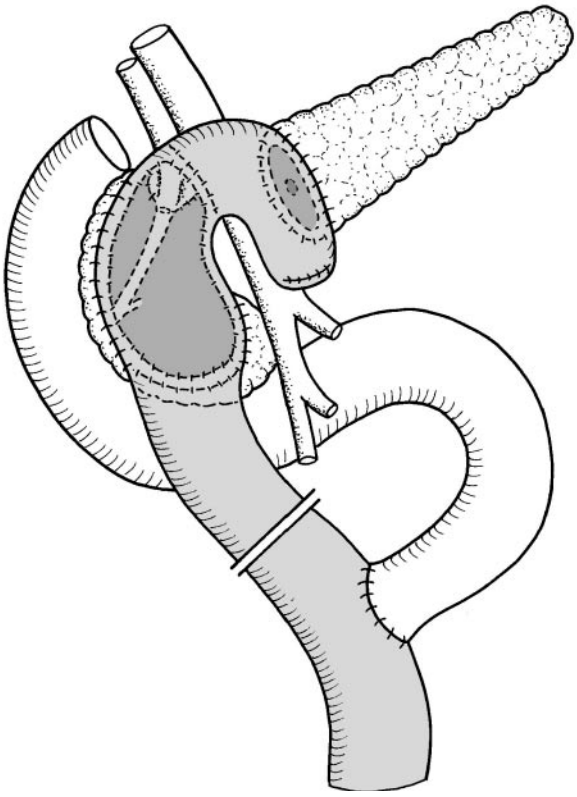
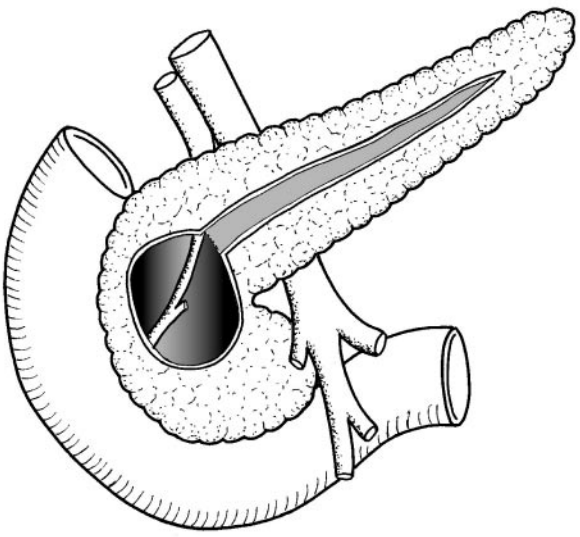


Figure 4. **Duodenum-preserving pancreatic head resection according to Frey: combinating of duodenum preserving pancreatic head resection and longitudinal drainage of the Wirsungian duct without division of the pancreatic body over the portal vein. (Modified from [60])**



original Beger procedure regarding safeness of the intervention and comparability of postoperative results.

Technical aspects of the duodenum preserving pancreatic head resection according to Beger and the Berne modification

Opening of the abdomen is performed by transverse or median upper laparotomy. Wide exposure to the pancreas is obtained by dissecting the gastro-colic ligament and opening of the bursa omentalis which allows the inspection and palpation of the whole pancreas. The intervention continues with mobilization of the right colic flexure and the Kocher mobilization of the duodenum and pancreatic head. After having identified the superior mesenteric vein at the lower border of the pancreatic body, the surgeon has to decide to perform original Beger procedure or not. In case of the Beger procedure, 4 stay sutures beside the expected resection line are placed at the lower and upper border of the pancreatic body to allow either gently lifting the pancreatic body away from the portal vein and prevent from excessive bleeding when dissecting the gland. The ventral wall of the portal vein is now gently dissected from the pancreatic body and the surgeon should be aware that especially in case of portal hypertension, this step represents the most delicate part of the intervention. Rupture of the fragile wall of the portal vein may lead to excessive bleeding and can hardly be controlled in this situation. After dissection of the pancreatic body with a scissor or scalpell, the neck of the gland is gently lifted away from the superior and portal vein tacking. Multiple stay sutures are placed all along the periphery of the pancreatic head which serve as reference points for the resection and will provide excellent hemostasis. Dissection of the pancreatic head starts right from the portal vein and is carried on onto the common bile duct. Care has to be taken to leave a 5-8 mm pancreatic tissue slice to the duodenum in order not to affect the blood supply of the duodenal wall (*Fig. 2*). We suggest holding the widely mobilized duodenum and pancreatic head in one hand to have optimal control on the extent of the resection and to avoid injury to the duodenal loop by preserving a cuff of intact pancreatic parenchyma. After the resection, it is essential to ensure meticulous hemostasis with PDS-5/0 single stitches both on the left pancreas and the remaining tissue of the pancreatic head. Reconstruction is performed with a Roux-en-Y loop with end-to-side pancreatico-jejunostomy and another side-to-side reconstruction between the remaining pancreatic head along the duodenum and the interposed jejunal loop. We always perform the pancreatico-jejunostomy in two layers with 5/0 PDS single stitches (*Fig. 3*).

The Berne modification spares the dissection of the pancreatic body from the portal vein. In this case, a single cavum results after the resection of the pancreatic head (*Fig. 5*), wich can be anastomosed side-to-side with a Roux-en-Y jejunal loop (*Fig. 6*).

If stenosis of the intrapancreatic part of the common bile duct cannot be resolved by decompression and resection of

Figure 5. **Berne modification of the original Beger technique, in this case with additional opening of the intrapancreatic common bile duct. Division of the pancreatic body over the portal vein is omitted. (Modified from [60])**

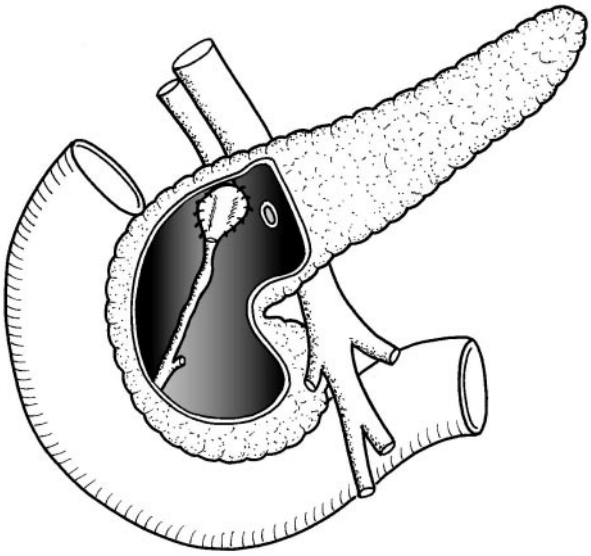
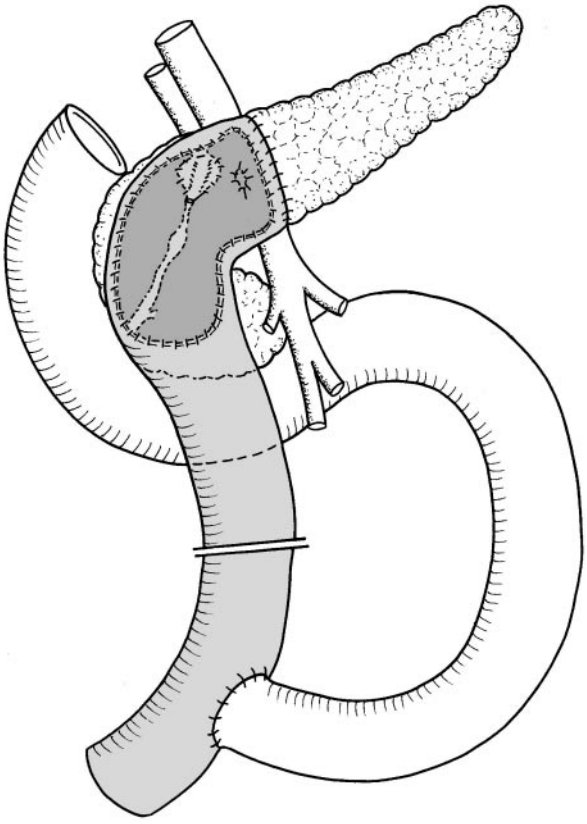


Figure 6. **Berne variation of duodeneum preserving pancreatectomy: reconstruction with Roux-en-Y jejunal loop and internal anastomosis of the opened intrapancreatic common bile duct. (Modified from [60])**



the surrounding pancreatic tissue, or if the intrapancreatic portion of the common bile duct is opened accidentally during pancreatic head resection, the wall of the opened bile duct is fixed with single stitches to the surrounding tissue like an opened door and is included in the same anastomosis (*Fig. 5*, *6*). In this case the gall bladder has to be removed to prevent from ascending cholangitis.

Conclusions

Simple drainage procedures are not sufficient to treat patients with chronic pancreatitis. Recent investigations clearly show that the head of the pancreas represents the pacemaker of this chronic inflammatory disease. Even in absence of a macroscopically enlarged pancreatic head, surgical procedures shall not be restricted to the body and tail of the gland. The aim of every surgical intervention to treat patients suffering from chronic pancreatitis and intractable pain should involve the resection of the inflammatory mass in the pancreatic head, if possible with minimal loss of intact pancreatic parenchyma and without collateral damage to neighbouring organs.

Excellent results regarding pain relief can be achieved with the classical Kausch-Whipple procedure and its pylorus preserving variant. However, these techniques have originally been developed for the treatment of malignancies and to our understanding represent over treatment in most of the cases. The rationale of these procedures is the complete resection of the inflammatory mass in the pancreatic head which can be better achieved by duodenum preserving techniques.

Several randomized trials show that compared with the Whipple procedure, the various techniques of duodenum preserving pancreatic head resection lead to excellent functional results and pain relief and are associated with a significantly reduced postoperative morbidity. Due to the largest experience, we favour the original technique according to Beger which we are actually comparing in a randomized trial with the Berne modification [61].

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Cardiac surgical treatment of the patients with renal insufficiency

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Abstract

Cardiac surgical treatment of the patients with renal insufficiency became more frequent necessity Also postoperative renal insufficiency occurs pretty often after cardiac surgery. That is in part a result of broadening of operative indications, which might concern patients with multiple diseases. Patients with renal insufficiency and coexistent heart diseases, patients with endocarditis and patients with renal insufficiency after cardiac surgery require the treatment of cardiac surgeons and nephrologists.

Heart diseases are the main cause of the mortality in the dialysis patients. Among the patients with renal diseases the cardiac surgeon most often receive long-term dialysis patients with coexistent heart diseases, who needs cardiac surgery (coronary artery by-pass grafting, valve operations). The amount of these operations increases, however it does not exceed 1% of overall number of cardiac operations. This group however, is very exacting and carries a high operative risk. Dialysis patients are exposed to increased risk of infection. 75% of them reveal infections in the form of sepsis. The presence of bacteria in the bloodstream increase the risk of infectious endocarditis. 6% of dialysis patients with IE require surgery.

The prevention of renal failure after cardiac surgery is also very important. Renal insufficiency occurs in 12% of patients after cardiac surgery with the use of extracorporeal circulation. Renal failure complicates postoperative course and is of high risk for the patient. The mortality due to acute postoperative renal failure, which requires hemofiltration, reaches 70%.

The proper cardiac surgical and nephrological management of renal insufficiency in patients selected for cardiac surgery as well as in patients with postoperative renal insufficiency is necessary to obtain good operative results.

Key words: renal insufficiency, cardiac surgery, heart diseases, dialysis.

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Introduction

Cardiac surgical treatment of the patients with renal insufficiency became more frequent necessity. Also postoperative renal insufficiency occurs pretty often after cardiac surgery. That is in part a result of broadening of operative indications, which might concern patients with multiple diseases. It is also in part negative effect of medicine progress. Which patients and which illnesses require the treatment both of professions; cardiac surgeon and nephrologist?

A – patients with renal insufficiency and coexistent heart diseases

B – patients with endocarditis

C – patients with renal insufficiency after cardiac surgery

Patients with renal insufficiency and coexistent heart diseases

Among the patients with renal diseases the cardiac surgeon most often receive long-term dialysis patients with coexistent heart diseases, who needs cardiac surgery. The amount of these operations increases, however it does not exceed 1% of overall number of cardiac operations. This group however, is very exacting and carries a high operative risk.

It has been shown that heart diseases are the main cause of

the mortality in the dialysis patients. 50% of all deaths in that group of the patients are caused by cardiac complications [1]. An increased tendency of occurrence of calcifications within the heart is the common nominative for every heart disease in the dialysis patients. Extraskeletal calcinosis occurs in 16% of the dialysis patients [2]. These calcifications have a close relationship to cardiovascular diseases and thrombotic events occurrence. Calcification of mitral ring, stenosis of mitral valve, stenosis of aortic valve, coronary artery disease (CAD) and peripheral atherosclerosis belonged to this group of illnesses. All of these diseases are life-threatening.

Secondary hiperparathyroidism is the main mechanism responsible for extraskeletal calcifications. It causes in inorganic phosphates and calcium plasma concentration increase. Extraskeletal calcifications take places mostly within the articular capsules. Histological structure of fibrous heart skeleton is similar, that is why calcium deposits within the heart [2]. Besides the tendency of calcification, higher mortality rate in long-term dialysis patients is related to coronary artery risk factors such as: hypertension, hyperlipidemia and bicarbonates metabolism disturbances. In addition all of these disorders accelerate the atherosclerosis. Surgical treatment of CAD, by using coronary artery by-pass grafting (CABG) becomes a standard procedure in long-term dialysis patients. It happens because of increased amount of the dialysis patients. This population is growing old and the age is also one of the risk factor for CAD.

Heart disease mortality is related to the age of the dialysis patients:

- in the group of patients within the age between 20-64 years cardiac deaths occur about 85/1000 patients per 1 year.
- in the group of patients over 65 years – about 131/1000 patients per 1 year.

Surgical treatment of ischemic heart disease in long-term dialysis patients has been applied since 30 years. The first CABG operation in the patient with renal insufficiency was performed by Menzoin in 1974. Taking into account that Favoloro for the first time initiated surgical treatment of ischemic heart disease in 1967, beginning of using that method in the dialysis patients was very early [1].

There are no uniform studies concerning modern cardiac surgical treatment results of long-term dialysis patients. The amount of studied patients is not yet enough to give the reliable conclusions. That is the result of small number of such the patients udergoing CABG every year. It is also very difficult to collect homogeneous group of these patients. Various operative qualification, operative and anaesthesiology technique progress and postoperative care changing through the years make difficult to collect homogeneous group for the study.

That is why there are no guidelines concerning operative qualification and operative methods in that group of patients yet.

Therefore all of the studies from the last years seemed to be too pessimistic when considering permanent treatment progress.

In 2000 Khaitan et al. in the group of 70 dialysis patients undergoing cardiac surgery showed hospital mortality rate of 14%, and complications rate of 50% [1]. 27 patients died within 5 years. The quality of life was improved only in 41% of the patients. There was no longer survival of the dialysis patients underwent cardiac surgery compared with those who had no surgery and the mortality rate for them was about 22% per one year in the United States. Khaitan observed the highest mortality rate in the group of the patients who had CABG together with valve operation [1]. There were no complications in the group of the patients after single elective operation, which were prepared for transplantation.

The high operative mortality, bad short and long-term results in some group of these patients, should indicate the patients who would take most advantage of cardiac surgical treatment:

- patients selected to kidneys transplantation,
- patients with advanced CAD or valvular heart disease in III / IV class for CCS or NYHA,
- patients with well or slightly abnormal left ventricular function,
- patients prepared to elective CABG or valve operation.

Invasive cardiology also finds its role in CAD patients undergoing dialysis. Koyanagi compared results of CABG and percutaneous transluminal coronary angioplasty (PTCA) with the stent implantation performed in the dialysis patients [3]. There were made about 2.5 grafts and 1.7 PTCA with the stenting per one patient. Angiography made after one month has shown 5% closed grafts (all arterial by-passes were patent) and 25% restenosis after stent PTCA. 70% of CABG patients and 18% of the patients after PTCA were free of angina pectoris after 5 years. It shows big difficulties in selecting the optimal treatment in that group of patients. While PTCA would not give sufficient results, and CABG in spite of the higher risk of postoperative bleeding and worse wound healing, remains the treatment of choice but when using at least one of the arterial graft. Survival after CABG and PTCA is decidedly longer than in cases of conservative treatment in long-term dialysis patients. Herzog observed, that 74% of medically treated dialysis patients died within two years after myocardial infarction (MI) [4]. If the patients were treated with CABG or PTCA procedure, two years survival was 56% and 48% respectively [4].

Valvular heart diseases are the following cardiac surgery problem after CAD in dialysis patients. In population of the patients without renal insufficiency CABG is performed in about 70-80% and valve operations in about 20-30%. Otherwise in the dialysis patients valve operations are performed more frequently, however CABG is still in majority [5]. Structural damage of the heart valves, which is more frequently presented in the dialysis patients, is combined with calcification of the fibrous heart skeleton and the valves. There are no differences in operative qualification procedure between the dialysis and non-dialysis patients undergoing valve operation.

Selection of the type of valve prosthesis should be done by the cardiac surgeon together with the nephrologist in respect of prosthesis durability and patient live expectancy. The durability of the biological prosthesis is estimated for 8-10 years. Biological prosthesis getting calcified after 10 years and after 15 years most of them need to be replaced, but this type of prosthesis do not need anticoagulation therapy. The durability of the mechanical valves is unlimited, but the need of anticoagulation treatment

carries 1-2% risk of bleeding and is associated with 0.2-0.4% of the risk of mortality per year.

The mortality rate for the dialysis patients exceeds 20% in the Unites States [1]. Cardiac surgeons in the U.S. prefer the biological prosthesis in spite of its limited durability, especially in the dialysis patients. The calcification and dysfunction of the biological prosthesis is getting faster in that group of patients. In Europe the mortality rate for the dialysis patients is estimated for about 10-12% per year. Therefore mechanical prosthesis are implanted more often in Europe. They have unlimited durability, but they have also higher possibility of infections and need permanent anticoagulation treatment, what could effect in higher complications percentage. Therefore, the decision about choosing the prosthesis should be made by the cardiac surgeon together with the nephrologist. All the time, this decision should be individualised and be depended on estimated time of survival.

One-year mortality rate for postoperative patients with implanted mechanical valve is estimated for 3-4%. It consists from:

- infections of mechanical valve (0.2-1.4 cases/1000 patients per one year) – they could have a bacterial background (mostly in the dialysis patients). They could be also a consequence of non-bacterial thrombotic endocarditis. Leucocytes infiltrate inside the thrombus and then ulcerating vegetation becomes. In the end of this process perivalvular abscess arises.
- thrombotic events (1.5-2.0 cases/100 patients per one year).
- bleedings (1-2% per one year) are responsibility for one-year mortality rate about 0.2-0.4%.
- prosthesis dysfunction – which concerns mechanical valve very rare. Mostly the biological prosthesis getting dysfunction. 60% of them are replaced after 15 years. The reoperation mortality is about 15%.

There are often more than one heart disease in population of the patients with renal dysfunction undergoing cardiac surgery. CAD and aortic valve disease belong to the same group of diseases related with degeneration process. These diseases often coexist together.

The occurrence of them is increased by the age. About 50% of the patients with aortic valve disease have more or less advanced coronary artery disease coexisted. CAD more often coexists with aortic valve stenosis than with aortic valve insufficiency. When symptomatical advanced CAD and aortic valve defect coexist together, the decision about the simultaneous operation is easy to take. When CAD patient selected for surgery has not very severe aortic valve disease, the decision about complex operation must be carefully done. The qualification for the operation is a little different for the patients with renal insufficiency than for the patients without it. The decision about the simultaneous operation is made according to: operative risk estimation of each of surgical procedures, estimated life expectancy, and estimated progress of the diseases. It is also important to estimate the risk related to mechanical valve prosthesis.

The risk of CABG is not so high, when left ventricular function is good, the risk is about 1-2%. The similar risk (2-4%) is for single valve replacement operation. The risk of simultaneous operation of valve and coronary arteries is higher (4-8%). However the risk of these operations made separately (CABG first, and valve replacement later) increases to 15%.

Trivial aortic stenosis (gradient of 25 mm Hg and aortic area surface higher than 1-1.5 cm^2) in the patients without renal dysfunction is not an indication for simultaneous valve replacement during CABG. On the other hand in dialysis patients the gradient increases not less than 10 mm Hg per one year. It means that dialysis patients with CAD selected for CABG with not significant aortic stenosis will require the valve replacement operation within five years.

There are not big differences in operative indications between the dialysis and non-dialysis patients. After previous CABG each valve operation is more difficult and could effect in the higher operative risk. It is a result of older age of the patients, progression of main disease and left ventricular function deterioration, which is the result of non-corrected valve dysfunction. The reoperation is also more difficult, because of adhesions. During the re-do surgery the risk of implanted grafts injury exists, especially concerning arterial graft – internal thoracic artery. The injury of mammary artery means an irreversible loss of the best arterial conduit.

Patients with infective endocarditis (IE)

Dialysis patients are characterised by an increased risk of occurrence of infection. It happens due to the defect in cellular immunity, neutrophil function, complement activation and the necessity to maintain vascular or peritoneal access. It is important that 75% of dialysis patients reveal infections in the form of sepsis, which is the cause of death in 25% of patients within the age 20-44, 15% – in the group of patients aged over 45 years [6]. The presence of bacteria in the bloodstream in the course of sepsis is a significant risk of infectious endocarditis occurrence.

Twenty years ago, IE occurred mainly in patients with valvular heart disease complicated with bacterial infection. Streptococcus was the main (60-80%) microorganism responsible for IE, most patients had rheumatic disease in their history. During the past years, the profile of patients falling ill with IE as well as the kind of most frequent pathogens were changed. The percentage of patients with rheumatic disease was diminished while the number of patients undergoing intensive and invasive therapy was elevated [7]. The increase in IE incidence in drug-addicts was also noticed.

Nowadays the main risk factor of IE becomes hemodialysis and the most frequent pathogen is Staphylococcus aureus. Immunosuppressive treatment, past operations, and infections of implanted artificial valves are also the risk factors. Streptococcus viridans is the second frequent pathogen responsible for IE. It should be noticed that while a 30-day-mortality in cases of IE is approximately 16%, with Streptococcus it reaches 50%.

IE in dialysis patients is a complication associated with high mortality. Patients with synthetic intravascular dialysis angioaccess are more likely to develop IE then patients with native arteriovenous fistulas, although IE in patients with fistula is characterised by higher mortality [6,8]. Prolonged antibiotic therapy is advised in all patients with IE. The patients with right-

sided IE, large bacterial vegetations in valves, diabetes mellitus, and increased leukocytosis should obtain special attention [9]. The frequency of IE occurrence is higher in patients undergoing hemodialysis (18%) than peritoneal dialysis (10.5%) [10]. The high risk of maintaining the vascular access caused trial of its removal in the course of IE therapy with consequent application of peritoneal dialysis [11]. That led to mortality lowering among patients dialysed peritoneally and treated with antibiotics to 8% in comparison with 55% among patients whose way of dialysis was not altered. Three patients out of both groups were operated on.

Approximately 6% of dialysis patients with IE undergo cardiosurgical treatment. The selection of an appropriate kind of valvular prosthesis is then of great importance. Recurrent bacteremia inclines to choose biological valves, which better tolerates infected environment. However, the implantation of such a valve to a young person can have the consequences of its fast degeneration after a few years and the risk of reoperation reaching 15%.

The prevention and management of postoperative renal failure

The prevention of renal failure after cardiac surgery and appropriate patient management, are important problems. It has been shown, that renal insufficiency occurs in 12% of patients after cardiac surgery with the use of extracorporeal circulation. Such a high percentage results from the complexity of surgical procedure, which requires long time of operation, significant blood loss, use of extracorporeal circulation and hypothermia. Renal failure complicates postoperative course and is of high risk for the patient. The mortality due to acute postoperative renal failure, which requires hemofiltration, reaches 70%.

There are many preoperative factors that predispose a patient to the occurrence of postoperative renal failure. These are: preoperative renal insufficiency, coexisting diabetes mellitus, advanced age, left ventricle failure, emergency operation, unstable angina pectoris, past strokes. It should be noticed that patients with normal renal function before the operation, rarely develop renal failure after operation. Creatinine level exceeding 1.5 mg% is an independent risk factor of postoperative renal failure, while higher than 2 mg% affects the increase in perioperative mortality. Preoperative insufficiency of renal function is reflected in each scale stratifying cardiosurgical operation risk. The scale most often used in Poland, the Euroscore scale, gives 2 points (in 12 points scale) in the case of creatinine level exceeding 2.3 mg%.

The pathogenesis of renal failure after cardiac surgery is complex. Blood, flowing through the cardiopulmonary by-pass, is exposed to thrombogenic artificial surface (drains, oxygenator) and the blood cells are damaged by the roller pump. It induces the whole body response in form of the complement activation, anaphylatoxins C3a, C5a initiating inflammatory response. The level of anaphylatoxin C3a is proportional to postoperative multiorganic damage. Whole body inflammatory reaction, leading to the increase in vascular permeability, induces accumulation of fluids in amount of 150 ml/kg of body weight. The second postoperative day is characterized by the increase of fluid in extracellular space by approximately 1/3 with body water increase by 13%. However, the unfavourable effect of CPB passes in most patients after few hours or days. Furosemid is capable of reversing CPB effect on renal flow. Dopamine in low doses has also synergic effect. Mannitol protects kidney function by increasing blood flow through the kidneys and glomerular filtration elevation.

Nonpulsative blood flow generated by cardiopulmonary by-pass, blood pressure drops below 50 mm Hg, resulting in tissue hypoperfusion are further potential risk factors of renal failure. Hemolysis always happens during cardiac operations. It is the result of a roller pump and sucking blood from the operative field. Free hemoglobin, which binds with plasma proteins: haptoglobin, hemopexin, and methemalbumin, is released during hemolysis. After the saturation of the binding sites, hemoglobin is filtrated by glomerules and reabsorption occurs through proximal renal tubules. The maintenance of urine alkalinity decreases possibility of hemoglobin precipitation in renal tubules.

A long duration of the aorta cross-clamping as well as cardioplegic cardiac arrest with high-potassium cardioplegic solution are other factors of renal failure. They can impair myocardial contraction and finally – cause renal hypoperfusion. Extracorporeal circulation duration, i.e. the complexity of operation, is a very important factor inducing these mechanisms. Thus, isolated procedures (CABG or valvular operations) are far less risky than complex ones [12].

It is assessed that blood and plasma flow through the kidneys, creatinine clearance, and urine secretion during the operation gradually decrease. The application of higher flows and pressures by the extracorporeal circulation pump can improve plasma flow, glomerular filtration, and sodium secretion. Hypothermia lowers glomerular filtration, renal blood flow, and osmolarity clearance but elevates filtration fraction and potassium secretion. One usually try to obtain hemodilution, which diminishes blood viscosity and enables linear blood flow through the systemic circulation and decreases the risk of central and systemic microembolism. The hematocrit lowering is tolerated to 20%; the level considered the borderline as far as oxygen transport is concerned. Hemodilution compensates unfavourable hypothermia effect by elevating renal blood by its viscosity decrease. The increased cortical flow affects urine secretion and free water clearance elevation, and thus counteracts the effect of vasopressin, secreted in response to hemodilution. High levels of aldosterone, vasopressin, and cortisol induce the kidneys to stop sodium and water and secrete potassium.

It should be added that there are many intraoperative factors that contribute to the increase of postoperative renal failure risk. These are: long extracorporeal circulation, bleeding, blood transfusions, low cardiac output, the necessity of catecholamines application, intraaortic balloon counterpulsation, and infection. Patients with preoperative renal insufficiency have their prognosis improved by forced diuresis; oliguria and nephrotoxic drugs avoidance is essential. If preoperative creatinine level exceeds 2.0 mg%, dopamine infusion in low doses is applied (2-5 μg/kg/min) before, during, and after the operation. Oliguria is treated with mannitol (12.5-25 mg) and furosemid intrave-

nously (10-100 mg, or higher doses). If there is no possibility of restoration of diuresis and hemodialysis is necessary, mortality reaches 70%.

While valvular heart diseases are operated on obligatory with the use of extracorporeal circulation, the coronary artery by-pass grafting, while operated without opening the cardiac chambers, could be performed on beating heart (OPCAB), if conditions are conducive. Approximately 20% of operations all over the world are performed without extracorporeal circulation. It was shown that OPCAB operations have lower percentage of renal complications, though not eliminated, than operations with extracorporeal circulation [12-14]. According to Provenchere, postoperative renal failure is much more related to the other factors than extracorporeal circulation, like advanced age, active endocarditis, recent (up to 48 hours) application of radiocontrast agent, poor postoperative cardiac function, and only then extracorporeal circulation [15]. Thus, most of the factors are preoperative ones and the surgeon has no influence upon them. However, the operative method is chosen by the surgeon and OPCAB operation is the one of choice in patients with increased risk of acute postoperative renal failure. Although we realize relatively small influence of operative method and the fact that not every operation can be performed in OPCAB.

Conclusions

In conclusion, heart diseases are the main cause of the mortality in the dialysis patients. Also postoperative renal insufficiency occurs pretty often after cardiac surgery. The proper cardiac surgical and nephrological management of renal insufficiency in patients selected for cardiac surgery as well as in patients with postoperative renal insufficiency is necessary to obtain good operative results.

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The non-invasive diagnosis of precancerous changes of stomach mucosa

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Abstract

Purpose: To detect the Helicobacter pylori (H.pylori)-induced gastric precancerous lesions leading to cancer formation, and to evaluate the possibility of non-invasive screening of dyspeptic patients to identify those having high risk of gastric cancer.

Material and methods: 178 consecutive H.pylori-positive dyspeptic patients after assessment of serum pepsinogen-1 (PG-1) and gastrin-17 (G-17) levels by enzyme immunoassay were examined with endoscopy and histology. The serologic and morphologic results were compared with estimating the sensitivity, specificity and prognostic values of the tests.

Results: There was statistically significant reverse dependence between the presence and severity of stomach mucosal atrophy (in antrum or corpus) and the proper serologic markers of stomach functional activity (G-17 or PG-1). On the other hand, the presence and the degree of intestinal metaplasia, dysplasia and gastric cancer did not correspond to the serum levels of G-17 or PG-1. The serologic method was quite sensitive in the diagnosis of non-atrophic and severe antral and corpus gastritis. Also, it was characterized by the high positive and negative prognostic values. Additionally, we have established the obvious advantage of the chromoendoscopy method in the diagnosis of intestinal metaplasia in the stomach epithelium.

Conclusions: The assays of serum G-17 and PG-1 levels can be offered as the screening tool for atrophic gastritis. The positive serologic results require further chromoendoscopic examination with mucosal biopsy to disclose the probable progression of atrophic process with development of intestinal metaplasia, dysplasia or gastric cancer.

Key words: gastric cancer, pepsinogen, gastrin, non-invasive screening, chromoendoscopy.

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Introduction

It has been precisely proven, that the cancer does not arise in healthy gastric mucosa. Various clinical and morphological studies have allowed the better understanding of pathological processes, which probably are precancerous. The malignant transformation of the normal gastric epithelial cells represents multistage process associated with progressing accumulation of genetic damages. According to the modern representations about stages of gastric carcinogenesis it is supposed that chronic Helicobacter pylori (H.pylori) infection is the trigger mechanism in 60-90% of gastric cancer cases. In a number of cases, H.pylori infection can be the only important etiological factor in development of gastric cancer. H.pylori has not been found out in a normal stomach, however it is frequently present in chronic gastritis, which progresses into atrophic gastritis, with subsequent development of intestinal metaplasia. This latter is seldom revealed in the absence of H.pylori infection. Molecular changes in H.pylori-infection, which are capable to result in the cancer development, are not established. There are no studies showing synthesis or secretion any mutagenous or carcinogenous substances by H.pylori. The current opinion is that H.pylori acts as a promotor rather than initiator of gastric carcinogenesis. H.pylori infection leads to the development of inflammatory reaction in gastric mucosa, with the production of reactive oxygen species by polymorphonuclear leucocytes and release of the cytokines by inflammatory cells, that results in the DNA damage and in stimulation of receptors triggering of cellular proliferation [1,2].

It is conventional to distinguish the precancerous (or

background) diseases and the precancerous changes of gastric mucosa. The first group represents pathological conditions, which under the appropriate circumstances can result in the development of gastric cancer (chronic gastritis, ulcer disease, polyps of a stomach, the resected stomach) i.e. associate with the increased risk of cancer development. The second group contains the morphologically proved changes of gastric mucosa reliably indicating on the development of the pathological process towards the malignant growth. These changes, under the recommendation a WHO (1978), were designated by the term "dysplasia". The possibility of progression of severe dysplasia to gastric cancer varies, according to the different data, from 8 up to 75%. The combination of precancerous conditions with precancerous changes of gastric mucosa really raises risk of cancer development. In the 1975 Correa proposed the consecutive line of events leading to gastric cancer development: normal mucosa – chronic active gastritis – chronic atrophic gastritis – intestinal metaplasia – dysplasia – carcinoma in situ. At present this cascade of changes associates with a trigger role H.pylori and is called "Correa's gastric precancerous cascade" [2,3].

The early detection of gastric precancerous changes, mainly atrophic gastritis, may be helpful in the prevention of gastric cancer or in the diagnosis of cancer at curable of stages. So, there is a critical need for valid diagnostic methods of stomach mucosal atrophy, that would be inexpensive and non-invasive, that is, suitable for screening of the large groups of the people.

Taking into account known interrelations between the morphological status and functional activity of gastric corpus and antral mucosa and the secretion of pepsinogen-1 and gastrin-17, respectively [4,5], we carried out a prospective study with the aim to detect the H.pylori-induced gastric precancerous changes, that are leading to cancer formation, and to evaluate the possibility of non-invasive screening of dyspeptic patients to identify those having high risk of gastric cancer.

Material and methods

The study was carried out according to updated Declaration of Helsinki in a group of 178 dyspeptic H.pylori-infected patients (109 female, 69 male, aged from 15 to 85 years, in average 59.8 ± 13.7 years), after informed consent for examinations. Any eventual medication with proton pomp inhibitors, H2 antagonists and NSAIDS were excluded for at least one month before examinations. Fasting serum Helicobacter pylori antibodies (Hp-Ab), serum levels of pepsinogen-1 (PG-1) and gastrin-17 (G-17) were assayed by enzyme immunoassay with Biohit GastroPanel® (Biohit Plc, Helsinki, Finland). According to the instruction of manufacturer, serum levels of PG-1 < 25 μg/l were estimated as markers of gastric corpus atrophy; serum levels of G-17 < 5 pmol/l were estimated as markers of gastric antral atrophy; serum levels of G-17 < 10 pmol/l in a combination with serum levels of PG-1 < 50 μg/l were estimated as markers of mild gastric corpus atrophy. Hp-Ab IgG titers were estimated as follows: < 32 EIU (EIU – enzyme immunoassay unit) – negative result; 32 - 44 EIU – doubtful result; > 44 EIU – positive result. The numerical meanings of assayed parameters were analyzed by the program GastroSoft® (Biohit Plc, Helsinki, Finland) enclosed to test-system Biohit GastroPanel®. On the basis of inserted data, the program composed the diagnosis in a view of the presence or absence of H.pylori-infection and mucosal atrophy, with the estimating of gastric cancer or peptic ulcer risk and with recommendations on the treatment according to Maastricht-2 consensus.

After getting the GastroSoft® diagnosis we have randomized 178 patients for the following study. The patients were undergone the upper gastrointestinal endoscopy with subsequent biopsy of the antral and corpus mucosa. For the increasing of the accuracy of endoscopic diagnosis we carried out the additional chromoendoscopy with the methylene blue staining allowing the detection of foci of intestinal metaplasia of gastric mucosa which were unrecognized by routine endoscopy. The biopsy specimens were stained with hematoxylin-eosin and PAS reaction in the combination with alcian blue at pH 2.5. The grade of stomach mucosal atrophy was estimated from 0 to 3 according to Houston visual analogous scale.

The statistical analysis was used to calculate the statistical significance of received data (Mann-Whitney criterion), the Spearman's correlation coefficient (r_s), the positive predictive value (PPV) and negative predictive value (NPV) of diagnosis by Biohit GastroPanel®.

Results

Of 178 patients, non-atrophic chronic gastritis (no antral atrophy and no corpus atrophy) was detected in 5 patients. The average meanings of serum PG-1, G-17 and anti-H.pylori IgG in this group were, respectively, 85.28 ± 35.07 μg/l, 14.44 ± 1.90 pmol/l and 85.68 ± 17.81 EIU. In all these cases there were no IM or dysplasia in stomach mucosal epithelium.

The morphological status of gastric corpus mucosa was compared to serum PG-1 levels (*Fig. 1*). The non-atrophic corpus mucosa was detected in 99 (55.62%) of 178 patients. The average meanings of serum PG-1 and anti-H.pylori IgG in this group were, respectively, 105.2 ± 5.7 μg/l and 68.76 ± 8.35 EIU. Mild atrophy of corpus mucosa was detected in 17 (9.55%) patients. The average meanings of serum PG-1 and anti-H.pylori IgG in this group were, respectively, 18.71 ± 0.95 μg/l and 77.21 ± 6.13 EIU. Moderate atrophy of corpus mucosa was detected in 36 (20.22%) patients. The average meanings of serum PG-1 and anti-H.pylori IgG in this group were, respectively, 11.91 ± 0.49 μg/l and 76.47 ± 5.04 EIU. Severe atrophy of corpus mucosa was detected in 26 (14.61%) patients. The average meanings of serum PG-1 and anti-H.pylori IgG in this group were, respectively, 6.92 ± 0.45 μg/l and 72.61 ± 4.72 EIU. The statistical analysis revealed the marked differences between the levels of PG-1 in non-atrophic and in atrophic corpus gastritis. The levels of PG-1 in mild, moderate and severe corpus atrophy were significantly lower ($p < 0.0001$) than in non-atrophic state. In turn, the levels of PG-1 in mild, moderate and severe corpus atrophy were differed significantly from each other ($p < 0.0001$).

The morphological status of gastric antral mucosa was compared to serum G-17 levels (*Fig.* 2). The non-atrophic

Figure 1. **Serum PG-1 levels compared to different degrees of corpus atrophy (dotted lines designate the mean values of PG-1, μg/l)**

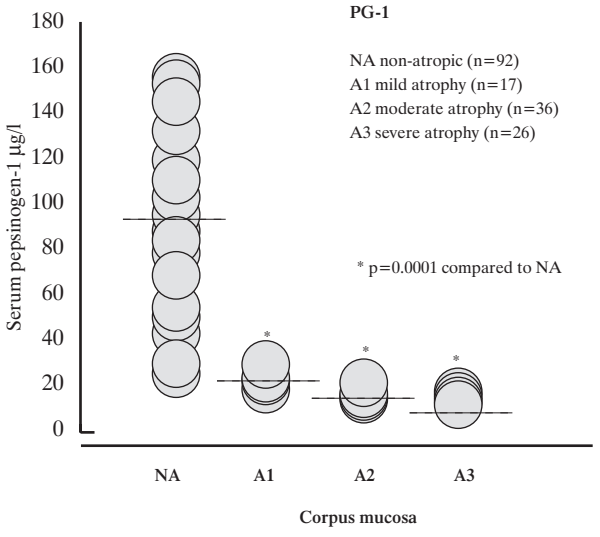

Figure 2. **Serum G-17 levels compared to different grades of antral atrophy (dotted lines designate the mean values of G-17, pmol/l)**

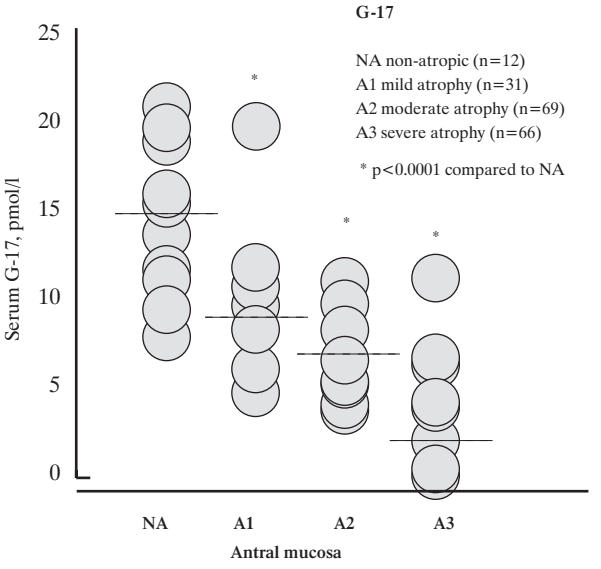

Table 1. **The comparisons of mean values of serum PG-1, G-17, Hp-Ab titers and the grade of IM**

	1 no IM (n=59)	2 mild IM (n=55)	3 moderate IM (n=41)	4 severe IM (n=23)	P_{1-2}	P_{1-3}	P_{2-3}	P_{1-4}	P_{2-4}	P_{3-4}
PG-1 (μg/l)	52.5 ± 7.74	66.07 ± 8.36	82.65 ± 10.72	53.08 ± 12.24	>0.05	<0.05	>0.05	>0.05	>0.05	>0.05
G-17 (pmol/l)	6.79 ± 0.55	5.48 ± 0.50	4.73 ± 0.67	5.11 ± 1.02	>0.05	<0.05	>0.05	>0.05	>0.05	>0.05
Hp-Ab (EIU)	81.23 ± 4.61	73.26 ± 4.81	72.09 ± 6.46	68.39 ± 7.94	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

antral mucosa was detected in 12 (6.74%) of 178 patients. The average meanings of serum G-17 and anti-H.pylori IgG in this group were, respectively, 14.57 ± 1.29 pmol/l and 62.29 ± 12.02 EIU. Mild atrophy of antral mucosa was detected in 31 (17.42%) patients. The means of serum G-17 and anti-H.pylori IgG in this group were, respectively, 8.91 ± 0.47 pmol/l and 78.25 ± 5.86 EIU. Moderate atrophy of antral mucosa was detected in 69 (38.76%) patients. The average meanings of serum G-17 and anti-H.pylori IgG in this group were, respectively, 6.40 ± 0.18 pmol/l and 74.08 ± 4.39 EIU. Severe atrophy of antral mucosa was detected in 66 (37.08%) patients. The average meanings of serum G-17 and anti-H.pylori IgG in this group were, respectively, 1.82 ± 0.26 pmol/l and 73.38 ± 5.38 EIU. The statistical analysis revealed the marked differences between the levels of G-17 in non-atrophic and in atrophic antral gastritis. The latter were significantly lower ($p<0.0001$) than in non-atrophic state. Moreover, the levels of G-17 in mild, moderate and severe antral atrophy were differed significantly from each other ($p<0.0001$).

We have detected intestinal metaplasia in 119 of 178 patients. The comparison of the grade of IM and the serum levels of PG-1, G-17 and anti-H.pylori IgG has showed the absence of the invariable, statistically significant dependence between these parameters (*Tab. 1*).

The dysplasia of stomach mucosal epithelium was detected in 113 patients. As in the cases of IM, we could not reveal the consistent and statistically significant dependence between the grade of dysplasia and the serum levels of PG-1, G-17 and anti-H.pylori IgG (*Tab. 2*).

Among 178 consecutive patients we have detected 6 cases of gastric cancer: 2 – early cancers and 4 – progressed tumors. The average meanings of serum PG-1, G-17 and anti-H.pylori IgG in these cases were, respectively, 46.73 ± 22.25 μg/l, 4.78 ± 2.086 pmol/l and 89.5 ± 11.7 EIU.

In general, our results have shown that there is a strong and statistically significant dependence between the presence and severity of stomach mucosal atrophy (antral or corpus) and the proper serologic markers (G-17 or PG-1) in H.pylori-associated chronic gastritis. On the other hand, the presence and the degree of IM, dysplasia and gastric cancer do not correspond to the serum levels of G-17 or PG-1. Thus, the serologic screening by means of Biohit GastroPanel® is useful for the selection of patients with stomach mucosal atrophy with subsequent thorough endoscopical and histological examination for the

Table 2. **The comparisons of mean values of serum PG-1, G-17, Hp-Ab titers and the grade of dysplasia**

	1 no dysplasia (n=65)	2 mild dysplasia (n=69)	3 moderate dysplasia (n=38)	4 severe dysplasia (n=6)	P_{1-2}	P_{1-3}	P_{2-3}	P_{1-4}	P_{2-4}	P_{3-4}
PG-1 (μg/l)	33.6 ± 5.41	79.98 ± 7.92	84.41 ± 10.98	71.88 ± 27.25	<0.05	<0.05	>0.05	>0.05	>0.05	>0.05
G-17 (pmol/l)	7.37 ± 0.46	5.28 ± 0.50	3.73 ± 0.66	4.53 ± 1.95	<0.05	<0.05	<0.05	>0.05	>0.05	>0.05
HpAb (EIU)	76.02 ± 4.15	77.65 ± 4.72	63.74 ± 6.32	91.27 ± 12.19	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

Table 3. **The correlations between results of different diagnostic methods**

Parameters	r_s
The detection of IM by endoscopy and by histology	0.41
The detection of IM by chromoendoscopy and by histology	0.92
The detection of antral atrophy by histology and the serum levels of G-17	- 0.75
The detection of corpus atrophy by histology and the serum levels of PG-1	- 0.71
The degree of stomach mucosal atrophy and of IM	0.21
The degree of stomach mucosal atrophy and of dysplasia	0.23
The degree of IM and of dysplasia	0.41

Table 4. **Sensitivity and specificity, PPV and NPV of Biohit GastroPanel® in diagnosis of stomach mucosal atrophy**

The degree of stomach mucosal atrophy (by histology)	Se	Sp	PPV	NPV
No antral atrophy	83%	95%	53%	99%
Mild antral atrophy	61%	84%	45%	91%
Moderate antral atrophy	67%	90%	81%	81%
Severe antral atrophy	89%	99%	98%	94%
No corpus atrophy	92%	97%	98%	91%
Mild corpus atrophy	71%	92%	48%	97%
Moderate corpus atrophy	72%	96%	81%	93%
Severe corpus atrophy	88%	97%	82%	98%

Table 5. **Sensitivity and specificity, PPV and NPV of routine endoscopy and chromoendoscopy in the diagnosis of IM**

Grade of IM	routine endoscopy				chromoendoscopy			
	Se	Sp	PPV	NPV	Se	Sp	PPV	NPV
no	98%	6%	35%	86%	94%	99%	98%	97%
mild	4%	98%	50%	68%	88%	88%	79%	94%
moderate	no data	no data	no data	no data	71%	95%	80%	92%
severe	6%	99%	33%	89%	82%	98%	82%	98%

possible development of precancerous or malignant changes in stomach mucosa.

Further we have compared the results of the serology, endoscopy and histology by means the Spearman's correlation coefficient (*Tab. 3*).

As shown in *Tab. 3*, the correlation between the results of routine endoscopy and histology was positive, but obviously weaker, than the correlation between the results of chromoendoscopy and histology. As it was expected, there was strong reverse correlation between the presence and the degree of stomach mucosal atrophy and the serum levels of the proper markers of its functional activity. The correlation between the degree of stomach mucosal atrophy and the subsequent morphological changes of mucosa – IM and dysplasia – was positive but quite weak.

The levels of sensitivity and specificity, PPV and NPV of Biohit GastroPanel® in diagnosis of stomach mucosal atrophy are presented in *Tab. 4*.

Thus, the investigated non-invasive method was quite sensitive in the diagnosis of non-atrophic and severe antral and corpus gastritis. Also, this method was characterized by the high PPV and NPV (except the cases of mild stomach mucosal atrophy).

Finally, we have carried out the comparison of sensitivity and specificity, PPV and NPV for routine endoscopy and chromoendoscopy in the diagnosis of IM (*Tab. 5*).

As a result, we have established the obvious advantage of a method of chromoendoscopy in the diagnosis of IM, that has doubtless diagnostic importance in the revealing of precancerous lesions of gastric mucosa.

Discussion

Gastric cancer remains the second biggest cause of cancer death worldwide [6]. In most cases, gastric cancer is preceded

for decades by persistent chronic active gastritis. This became clear in the 1960s and 1970s, when endoscopic biopsy sampling became possible and various excellent cohort studies on the dynamics of gastritis were performed, in particular in Scandinavia and the Baltic States. It appeared that the anatomy and function of the gastric mucosa normally remained unchanged throughout life, unless chronic active gastritis was present [7]. The most common cause of gastritis was unknown at that time, but later it appeared to be Helicobacter pylori colonization. Chronic H. pylori gastritis eventually leads in more than half of the affected subjects to gradual loss of glandular structures with its specialized cells and a collapse of the reticulin skeleton of the mucosa, a condition of atrophic gastritis [8]. As a result, the glandular layer of the mucosa becomes thinner, and glands are replaced by fibrosis and intestinal metaplasia. The major clinical importance of this condition is that it significantly increases the risk for the intestinal type of gastric cancer. This risk may be elevated up to 90 fold in subjects with severe atrophic gastritis throughout the complete stomach [9]. The annual incidence of gastric cancer among patients with atrophic gastritis varied in cohort studies between 0.3 and 1.0% [10]. This explains the interest in the diagnosis of atrophic gastritis. At present, there is a broad spectrum of questions related to the diagnosis of critical stages of gastric carcinogenesis: gastric epithelial atrophy, intestinal metaplasia and dysplasia. Therefore, it is extremely important to recognize among the dyspeptic patients those, who have very high risk of gastric malignant changes and who require dynamic surveillance, with the purpose of early diagnosis any preneoplastic changes in the stomach mucosa. Atrophic gastritis is a serious disease, which often does not receive much attention. The relationship between gastritis, atrophic gastritis and other diseases of the stomach is based on the fact that the infection and atrophy alter the physiological functions of the stomach and influence the growth and growth control of the epithelial cells in the stomach. These consequences vary, depending on whether the changes of the gastric mucosa caused by gastritis are located in the antrum or the corpus or both [11].

The most accurate diagnostic method of the gastrointestinal tract diseases is endoscopy with subsequent biopsy, which should be made in all patients with the presence of clinical symptoms. However, because of patchy character of atrophic changes in the stomach mucosa, some histological examinations could give false, negative results. Beside this, the biopsy is an expensive and labor-consuming method of research [12], so it can not be carried out in all patients in succession. In the contrary, due to invasiveness of biopsy, it is expedient to make only for monitoring of precancerous changes in the stomach mucosa. Therefore a screening method for the qualification of the patients group, recommended to biopsy is necessary. Such a method should be capable to reflect objectively the functional condition of the stomach mucosa, and hence, its morphological status.

It has been known for over two decades that the atrophic gastritis of the corpus and fundus of the stomach can be determined reliably by measuring the serum pepsinogen-I (PG-1) or the PG-1/PG-2 ratio from a blood sample [13-15]. However, it has not been possible to determine from a blood sample the types of atrophic gastritis in which the atrophic changes are located solely in the antrum. The GastroPanel® serum test also enables the determination of atrophic gastritis of this antrum-limited subtype.

Group I pepsinogens are synthesized solely in the oxyntic glands and mucous neck cells of the gastric body. On the other hand, pepsinogens of group II are uniformly synthetized in the glands of the entire stomach and to some extent also in the Brunner glands in the first part of the duodenum. The majority of the pepsinogens are secreted into the lumen of the stomach, where they are activated to the pepsin. A small proportion of the pepsinogens leaks into the blood circulation. In the case of atrophic corpus gastritis, the level of serum pepsinogen-1 decreases whereas the level of pepsinogen-2 remains stable or decreases slightly. The level of serum pepsinogen-1 or the ratio of serum pepsinogen-1 to pepsinogen-2 reflect with high reliability the number of cells and the number of oxyntic glands in the corpus area of the stomach, i.e. they reflect the degree of atrophy of the corpus mucosa. As the severity of the atrophic corpus gastritis corpus increases the level of serum pepsinogen-I or the PG-1/PG-2 ratio decreases [14-16].

Gastrin is synthesized in G-cells, which have been found in the gastric antrum. The most important stimulators of gastrin secretion into the circulation are: the vagal drive, the gastrin releasing peptide hormone (GRP; bombesin) and intraluminal factors, such as the stretching of the antrum or the protein-rich contents (diet) in the stomach. A pH-level below 2.5 lowers the secretion and release of gastrin from antral G-cell. The gastrin secreted in the antrum is over 90% of type G-17, whereas the gastrin secreted by the duodenum is primarily of type G-34. The fasting serum gastrin is primarily in the form of G-34, but the proportion of the type G-17 increases after the dietary stimulus. The secretion of gastrin-17 can be studied with a simple protein stimulation test. First, a blood sample is taken after fasting, after which the patient eats a protein-rich meal. The maximum increase in the serum level of gastrin-17 can be seen within 20 minutes. If the serum gastrin does not increase as a result of protein or other physiological stimulations it is an indication of the loss of gastrin secreting G-cells, i.e. an indication of the atrophy of the antrum mucosa. It is possible to make indirect conclusions regarding the antrum mucosa status by simultaneous assays of the serum gastrin and gastric acid output. In the cases with atrophic antral gastritis and loss of antral G-cells, serum gastrin remains low although the stomach is achlorhydric or hypochlorhydric [17].

Several research groups have renewed the interest in serology for atrophic gastritis by combining gastrin and pepsinogens with Helicobacter serology. Väänänen and colleagues present a smart algorithm for the differentiation in both antrum and corpus between atrophic and non-atrophic gastritis [18]. The algorithm was tested in a cross-sectional study correlating gastric mucosal histology with H. pylori IgG serum antibodies, serum PG-1 levels, and fasting and postprandial serum gastrin-17 levels. It appeared that in roughly 80% of the 404 cases tested, histology and serology matched a similar diagnosis. Sixty (15%) of the 404 subjects had atrophic gastritis, 6 (1%) had previously undergone antral resection, and 340 (84%) had a non-atrophic gastric mucosa either with or without inflammation. In this population with a rather low prevalence of atrophic gastritis, the negative predictive value of the serology

panel was 93-97% and the positive predictive value was 64-75%. For these calculations, the authors combined all subjects with atrophic gastritis of the antrum, the corpus, or both. The data, however, show that the serology panel performed much better in diagnosing atrophic gastritis in the corpus than in the antrum. In only 19 (50%) of the 38 patients diagnosed by serology as having antrum atrophic gastritis was this condition confirmed by histology.

This revival of interest in the serological testing of the condition of the gastric mucosa is of importance, given the fact that H. pylori eradication may cure gastritis and help to prevent further progression of gland loss. It is likely that this may also reduce the risk for gastric cancer [19], although many more data on this are needed. Screening and treatment of H. pylori infection may in theory be cost-effective for the prevention of gastric cancer [12].

Sipponen et al. [20] have recently shown, that the simultaneous detection of serum concentrations of PG-1 and G-17 and Hp-Ab titers is an effective method for non-invasive screening and diagnosis of atrophic gastritis using the blood samples of the patients.

In our present study, the use of the test-system GastroPanel® has allowed to receive statistically significant differences between the serum concentrations of PG-1 and G-17 depending on a degree of a stomach mucosal atrophy. We have obtained quite satisfactory meanings of the PPV and NPV of GastroPanel® test in the revealing the atrophic state of stomach mucosa. Moreover, this test was sufficiently sensitive and specific as it was proven by chromoendoscopy and histology.

Thus, our study has confirmed the usefulness of the test-system GastroPanel® as a "serologic biopsy" for authentic and non-invasive diagnosis of atrophic changes of a stomach mucosa in the patients with dyspepsia, associated with H. pylori infection. Beside this, we managed to show the advantage of a chromoendoscopy method prior to routine endoscopy in the diagnosis of intestinal metaplasia. Now is the time, almost a decade after the conclusion of the World Health Organization (WHO) that H. pylori is a class I carcinogen [21], to use this serology in further studies in selected and general populations. This will allow evaluation of the feasibility of screening and treatment for gastritis and prevention of gastric cancer, a cancer that is much more common than many other disorders for which screening and prevention have long been accepted in many populations.

Conclusions

The non-invasive detection of gastric mucosal atrophy by means of enzyme immunoassay with assessment of G-17 and PG-1 levels can be offered as the screening tool for gastric precancerous conditions. On the other hand, this method does not allow to diagnose the intestinal metaplasia and/or dysplasia in stomach mucosa. Therefore, the results of the serological screening indicating the stomach mucosal atrophy require carrying out the chromoendoscopy with subsequent mucosal biopsy, for revealing probable progression of atrophic process with the development of intestinal metaplasia, dysplasia or gastric cancer.

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Is a bile reflux an additional cancerogenic factor in peptic ulcer, associated with Helicobacter pylori infection?

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Abstract

Purpose: **It's well known that Helicobacter pylori (H.p.) infection plays a leading role in gastric cancer in 70% of patients. A role of bile acids reflux in the stomach as a potentially cancerogenic factor is disputable. As the influence of both factors for malignancy, is still unknown, it was the aim of our investigations in peptic ulcer (PU) patients complicated with duodenogastric reflux (DGR).**

Material and methods: **79 PU patients (49 men, 27 women, aged 18 to 61, middle age – 41.8 yrs) were observed; DGR was found in 40 of them. For all patients endoscopie examination with antral and fundal parts of the stomach mucosa biopsy were performed. The morphological picture was estimated by Dixon's scale.**

Results: **The similar H.p. colonization level of the stomach mucosa independently of DGR presence was shown. A bile reflux co-exists with diminished acute and chronic inflammation activity and atrophy level. The quantity of the patients with complete intestinal metaplasia did not depend of DGR (32.5% vs. 33.3%), but in the patients with DGR it developed 16 years earlier (42.8 and 58.7 yrs, $p < 0.05$).**

Conclusions: **The combination of a bile reflux and H.p. infection may to provide progression to gastric cancer not by traditional stepwise: inflammation – atrophy-metaplasia.**

Key words: duodenogastric reflux, Helicobacter pylori, inflammation, atrophy, intestinal metaplasia.

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Introduction

Yet 10 years ago a Working Group of the World Health Organization (WHO) and the International Agency for Research on Cancer concluded that Helicobacter pylori (H.p.) is a class 1 cancerogen, and it means that the organism plays a causal role in the development of gastric cancer [1]. The prospective studies have confirmed the association between H.p. infection and the subsequent development of gastric cancer [2-4]. Now it is well known that more then 70% of gastric cancers are associated with H.p. infection. H.p. infection induces an infiltration with neutrophils and macrophages – a marker of acute and chronic inflammation of gastric mucosa. The resulting damage of this mucosa is followed by atrophic gastritis and intestinal metaplasia. Now 3 ways of mutagenesis with duodenal ulcer disease (DUD) duodenogastric reflux (DGR) H.p. participation are proposed. The 1st is inflammation-related; the 2nd is based on mitotic errors, and the 3rd underlines importance of dietary mutagens. The direct stimulation of apoptosis by H.p. is not excludes [5].

In 35-70% patients with complicates principal disorder [6]. A role of bile acids in stomach reflux as a potentially cancerogenic factor is under discussing [7]. So, Robbles at al. [8] considers that gastritis symptoms are more caused with DGR then H.p. status. But it's still unknown the influence of both this factors for malignancy.

The aim of our investigations was to value a compatible influence of H.p. infection and a bile factor on the inflammation, atrophy and metaplasia of gastric mucosa by observation of patients with DUD and DGR.

Material and methods

79 DUD patients (49 men, 27 women, aged 18 to 61, middle age – 41.8 yrs) were observed. In all patients endoscopie investigation with antral and fundal part of the stomach mucosa biopsy was performed. The DGR was confirmed by the bile

***Table 1*. Histological changes of gastric mucosa in the duodenal ulcer disease (DUD) patients with (DGR+) and without (DGR-) duodenogastric reflux by Dixon's score (score, M±m)**

Histological changes	Antrum (n=40)			Fundal (n=39)		
	DGR+	DGR–	p	DGR+	DGR–	p
H.p. colonization	2.38±0.10	2.53±0.10	n.s.	1.10±0.10	1.10±0.10	n.s.
Polymorphonuclear neutrophils infiltration	2.45±0.09	2.72±0.08	<0.05	1.15±0.10	1.08±0.10	n.s.
Mononuclear cells infiltration	1.95±0.11	2.22±0.08	<0.05	1.45±0.09	1.75±0.09	<0.05
Atrophy	1.5±0.13	2.08±0.12	<0.001	1.15±0.11	1.81±0.11	p<0.001

H.p. – Helicobacter pylori
n.s. – no significant

presence at the endoscopic examination, pH fluctuation >4 during 24-hours pH-metry and histological picture of reflux gastritis. The morphological signs of acute inflammation, chronic inflammation, atrophies and metaplasia were estimated by Dixon's scale [12]. For all patients the H.p. infection status, using CLO-test, histology and culture methods was assessed.

Result

The results show (*Tab. 1*) that in patient with DGR, H.p. colonization level (HpCL) in antral part of the stomach was 2.4±0.10 points, in fundal – 1.1±0.10. Polymorphonuclear neutrophils infiltration level (PNIL) was 2.4±0.09 and 1.1±0.10 respectively. Mononuclear cells infiltration level (MCIL) was 2.0±0.11 and 1.4±0.09. Atrophy level (AL) was 1.5±0.13 and 1.2±0.11. Complete intestinal metaplasia (CIM) was assessed to be 7.5% and 2.5% of patients and incomplete intestinal metaplasia (IIM) – in 32.5% patients (in antrum only).

In patients without DGR: HpCL in antral part of the stomach was 2.5± 0.10 and in fundal one 1.1±0.10 respectively; PNIL 2.7±0.08 and 1.1±0.10; MCI 2.2±0.08 and 1.8±0.09; AL 2.1±0.12 and 1.8±0.11. CIM was observed in 8.3% of patients (in fundal only), IIM – 33.3% of patients (in antral part of the stomach only). This data show similar H.p. colonization level of the stomach mucosa independently of DGR presence (p>0.05). The intensity of acute and chronical inflammation in gastric antrum mucosa was less in patients with DGR than without it, as well as the intensity of chronical inflammation (mononuclear cells infiltration score) in gastric fundum DGR. The atrophy score as in antrum so in fundal part of the stomach was less too.

Discussion

The role of bile as a potentially cancerogenic factor was studied in many cases. Study on animal's model [9] had show that H.p., especially combined with bile, has an influence on cell kinetics, contributing to the development of gastric cancer. Other histological studies of gastric mucosa suggest that intragastric bile acids may be implicate in premalignant histological changes of gastric mucosa and thus play a part in gastric carcinogenesis [10].

The controversial studying had demonstrated comparable low increase of duodengastric reflux concentration in proximal gastric ulcer and distal gastric carcinoma. That gives no evidence for a main role of reflux in the pathogenesis of gastoduodenal ulcer [11]. But in our results it seems a paradox that a bile reflux promotes diminished acute and chronical inflammation activity and atrophy level of gastric mucosa in H.p. infected patients. Although the quantity of patients with complete intestinal metaplasia does not depend on DGR (32.5% and 33.3%), but in patients with DGR it develops 16 years earlier. The middle age of metaplasia for patients with DGR was 42.8 ±3.0 and 58.7±2.2 yrs without it (p<0.05). It means that a bile reflux could hasten the development of incomplete intestinal metaplasia in H.p. infected patients. Another authors also underlines the danger of both factor (a bile and H.p.) for cancerogenesis [12-14].

It is difficult to explain this result, but there is all reasons to think that the combination of a bile reflux and H.p. infection provides progression to cancer by not traditional stepwise according to Correa but alternative way suggested by Sipponen [15].

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Gastric acid and salivary bicarbonate. Is there a relationship in duodenal ulcer patients?

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Abstract

Purpose: Since saliva protects the oesophageal and oral mucosa against hydrogen ions, the aim of the study was to establish the relationship between the secretion of gastric acid and salivary bicarbonate.

Material and methods: The study involved 43 Helicobacter pylori positive duodenal ulcer patients receiving: 1. omeprazole alone (O), 2. omeprazole and amoxicillin (OA) or 3. omeprazole, amoxicillin and tinidazole (OAT). In each study group the examination was performed twice, before and at the end of a two-week treatment, both under basal conditions and during a gastric secretory test with pentagastrin. Concentrations of gastric hydrogen ions and salivary bicarbonate were evaluated by the titration method.

Results: In all therapeutic groups analysed separately, the secretion of gastric acid as well as salivary bicarbonate decreased at the end of the treatment, however only in OA and OAT groups the differences in bicarbonate reached statistical significance. As the changes in the concentration and output of both salivary bicarbonate and gastric acid had the same direction, the three therapeutic groups (O, OA, OAT) were subjected to combined analysis. It showed that under basal conditions and during stimulation with a gastric catheter or catheter and pentagastrin, bicarbonate concentration and output were higher before than at the end of the treatment. However, no direct correlation between gastric acid secretion and salivary bicarbonate was found in groups subjected to either separate or combined analysis.

Conclusions: The results of our study provide evidence for the partial involvement of hydrogen ions of gastric origin in the regulation of salivary bicarbonate secretion in duodenal ulcer patients.

Key words: duodenal ulcer, gastric acid, salivary bicarbonate.

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Introduction

Saliva exerts many physiological actions in the oral cavity and oesophagus. A crucial one is the protection of oesophageal mucosa against refluxed gastric acid [1,2]. Attempts have been made several times to determine the correlation between the secretion of gastric acid and saliva, but the results have not been conclusive; the correlation was found to be either positive or none [3,4]. The effect of gastric acid on salivary secretion may occur as a result of gastroesophageal reflux and reflex regulation of salivary secretion associated with stimulation of pH-sensitive receptors located in the lower oesophagus [1,5,6]. In duodenal ulcer patents, this regulation is responsible for 25% of total salivary secretion [7].

In the neutralisation of gastric acid refluxed to the oesophagus bicarbonate ions play an essential role; they originate either from the oesophageal submucosal mucus glands or saliva. Since in this action saliva is most important [1,2], the aim of the study was to establish the relationship between the secretion of gastric acid and salivary bicarbonate.

We designed our study for duodenal ulcer patients as their gastric acid secretion is relatively high and additionally it can be easily decreased or increased, using omeprazole and pentagastrin. By distinguishing therapeutic groups of similar initial (pH = 2) but different final gastric acidity (pH = 4 and pH = 7) [8,9], we expected to answer the question what gastric acidity, if any, is involved in this regulation.

Material and methods

Patients

Forty-three duodenal ulcer patients (33 men, 10 women; mean age 38 years, range 20-64 years; 23 smokers and 20 non-smokers) entered the study. The qualifying criteria included: duodenal bulb ulcer 5-10 mm in diameter, H. pylori infection of gastric mucosa and normal routine laboratory tests. The exclusion criteria were as follows: ulcer related to non-steroidal anti-inflammatory drugs, treatment with other drugs than antacids within the previous two weeks, gastro-duodenal surgery, complicated duodenal ulcer, oesophagitis, pregnancy and lactation, salivary gland diseases and poor general condition.

Study groups

Patients were allocated to one of the three therapeutic groups receiving a two-week oral treatment with: 1. omeprazole alone (2x20 mg) (15 subjects), 2. omeprazole (2x20 mg) and amoxicillin (2x1000 mg) (14 subjects) and 3. omeprazole (2x20 mg) in combination with amoxicillin (2x1000 mg) and tinidazole (2x5000 mg) (14 subjects).

Saliva collection

Saliva was collected twice, before treatment (under basal conditions and during a gastric secretory test with pentagastrin) and at the end of treatment (day 13 – saliva collection under basal conditions; day 14 – collection during a gastric secretory test with pentagastrin). Saliva collections at the end of the treatment were performed 12 hours after the evening doses of drugs, starting always at 8.00 a.m. On the day of the examination the subjects remained in a fasting state, having refrained from eating since the previous evening and from drinking since midnight. They were required to refrain from smoking in the morning hours prior to saliva collection. On examination, subjects were in a sitting position, expectorating the saliva to volumetric caps every 15-30 seconds. Each time, a total saliva collection period was 135 minutes. As saliva secretion during the first few minutes varied greatly, the initial 15-min fraction was discarded. The remaining part was mixed carefully and frozen at a temp. of -25^{0}C.

Collection of gastric contents

The tip of a gastric catheter (5 mm in diameter) was inserted transnasally to the antral region of the stomach. During the examination all subjects were in a sitting position. Residual gastric contents were removed for 15 min and discarded. Then, the contents were collected for 60 min without additional stimulus and the collection was continued for another 60 min following a subcutaneous administration of pentagastrin (Peptavlon, Zeneca) at a dose of 6 μg/kg. Gastric secretion was assessed twice, before and at the end of the two-week treatment. In subjects in whom saliva was collected under basal conditions, gastric acid secretion was not monitored. For them we used the results of gastric secretory test performed a day later.

Assay of salivary bicarbonates

One ml of saliva, previously unfrozen at room temperature was used to determine bicarbonate concentration. Measurements were performed using the titration method [10], with a pH-meter (HI-9321, Hanna Instruments) scaled on standard buffers. At first, it was found that freezing and unfreezing had no effect on bicarbonate concentration, and the values did not differ from those obtained in the freshly collected saliva. Salivary bicarbonate concentration was expressed in μmol/ml, output in μmol/min.

Assessment of gastric secretion

Gastric secretion of hydrogen ions was determined using the titration method. Basal and maximal acid secretion were assessed directly after the collection with 0.1 MNaOH solution, starting from the initial pH to pH 7.0 [11]. The secretion of gastric juice was expressed in ml/hour, concentration of hydrogen ions in pH units and output in mmol/hour.

Assessment of H. pylori infection

During diagnostic gastroscopy which preceded qualifying patients for the study, 6 gastric mucosa specimens, 3 specimens from the gastric antrum and 3 from the gastric body were collected. In one specimen of each series, H. pylori infection was determined with a rapid urease test (CLO-test), in the remaining two, the histological method was used (staining with Giemsa method); the outcome of both tests for H. pylori was positive in all subjects.

Statistical analysis

The results were expressed as means ± S.E. The Wilcoxon's test for paired values was used for statistical analysis. The level of significance was accepted for $p < 0.05$.

The study was approved by the Bioethics Committee, Medical University of Białystok. All the subjects gave informed consent to the participation in the study.

Results

Under basal conditions, concentration and output of salivary bicarbonate were lower at the end of the treatment than at the baseline; however, significant differences were found only in patients treated with OA regimen (9.23 ± 1.04 vs 6.23 ± 0.60 μmol/ml and 3.38 ± 0.60 vs 2.29 ± 0.38 μmol/min, $p = 0.007$ and $p = 0.021$, respectively) and with OAT regimen (7.24 ± 0.54 vs 5.92 ± 0.49 μmol/ml and 3.75 ± 1.04 vs 2.52 ± 0.70 μmol/min, $p = 0.006$ and $p = 0.011$, respectively) (*Fig. 1* and *2*). In the omeprazole group, the differences were not significant.

During the first hour of the gastric secretory test (the catheter tip inserted transnasally to the stomach) no statistically significant differences were found in the concentration and output of salivary bicarbonate between the baseline and the end of the two-week treatment in all study groups (*Fig. 1* and *2*).

In the second hour of the gastric secretory test (the catheter inserted to the stomach with a simultaneous pentagastrin stimulation), bicarbonate concentration at the end of the treatment decreased as compared to the baseline, but only in OA and OAT groups the differences were significant (8.75 ± 1.19

Figure 1. **Salivary bicarbonate concentration under basal conditions and during a gastric secretory test with pentagastrin before (1) and at the end (2) of a two-week treatment (means ± S.E.)**

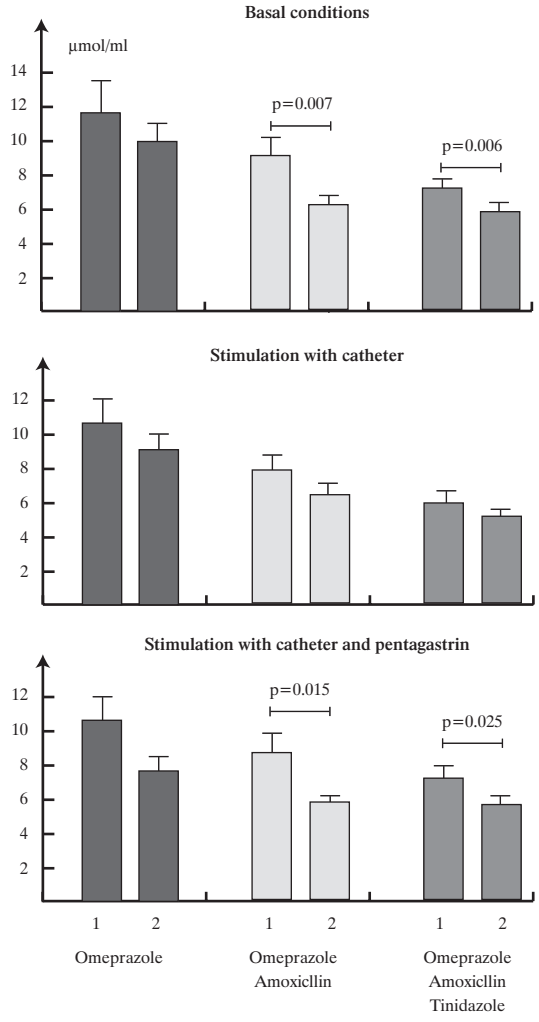
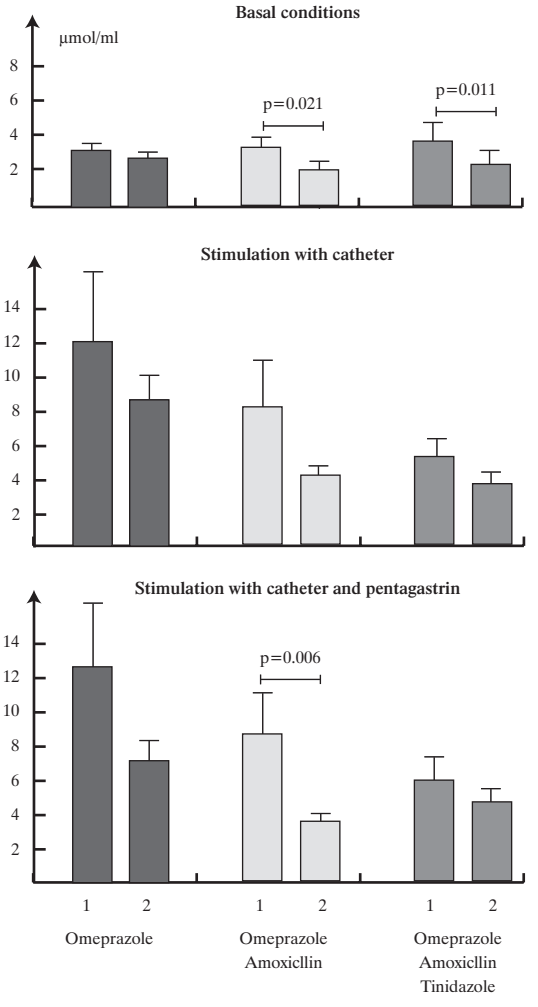


Figure 2. **Salivary bicarbonate output under basal conditions and during a gastric secretory test with pentagastrin before (1) and at the end (2) of a two-week treatment (means ± S.E.)**



vs 5.75 ± 0.38 μmol/ml and 7.41 ± 0.68 vs 5.65 ± 0.56 μmol/ml, p = 0.015 and p = 0.025, respectively). At this time bicarbonate output was also lower but the difference was significant only in OA-treated group (8.08 ± 2.49 vs 3.23 ± 0.52 μmol/min, p = 0.006) (*Fig. 1* and *2*).

At the end of the treatment, in the first and second hour of the gastric secretory test, the volume of gastric content was lower, while pH was higher; the highest pH was observed in the group treated with omeprazole alone (*Tab. 1*). The output of hydrogen ions was lower at the end of the treatment in all therapeutic groups; this concerned both the first and second hour of the gastric secretory test.

As the changes in the concentration and output of both salivary bicarbonate and gastric acid had the same direction, the three therapeutic groups were subjected to combined analysis. It showed that under basal conditions and during stimulation with a gastric catheter or catheter and pentagastrin, bicarbonate concentration and output were higher before than at the end of the treatment (*Tab. 2*). However, no direct correlation between gastric acid secretion and salivary bicarbonate was found in groups subjected to either separate or combined analysis.

Discussion

The present findings indicate that in duodenal ulcer patients gastric antisecretory therapy may contribute to the decrease in salivary bicarbonate secretion. The saliva collection performed 12 hours after taking the final dose of omeprazole may exclude a direct effect of the drug; its serum concentration 8 hours after administration cannot be determined [12]. Serum amoxicillin concentration is also short-lasting (approximately 4 hours) [13], while tinidazole concentration is high not only during the treatment but also for a few consecutive days following its termination [14]. The same direction of changes in the concentration and output of salivary bicarbonate at the end of the treatment in all therapeutic groups suggests, however, that the direct effect of tinidazole on the examined saliva indices is insignificant as well.

Table 1. **Gastric secretion (mean ± S.E.)**

		O		OA		OAT	
		1	2	1	2	1	2
Basal	volume (ml/h)	76.6 ± 13.7	44.8 ± 6.6 [a]	90.6 ± 15.3	37.4 ± 4.4 [b]	76.9 ± 16.8	34.5 ± 3.5 [b]
	pH	2.12 ± 0.28	7.21 ± 0.34 [b]	1.80 ± 0.43	4.26 ± 0.45 [a]	1.76 ± 0.35	3.65 ± 0.55 [a]
	output H^+ (mmol/h)	4.63 ± 1.19	0.19 ± 0.19 [a]	4.54 ± 0.97	0.88 ± 0.17 [a]	4.28 ± 1.12	0.59 ± 0.15 [a]
Pentagastrin	volume (ml/h)	195.6 ± 15.5	93.9 ± 24.1 [a]	184.9 ± 18.9	80.9 ± 15.6 [a]	212.9 ± 14.3	67.2 ± 12.1 [b]
	pH	1.43 ± 0.13	4.89 ± 0.48 [b]	0.98 ± 0.10	2.59 ± 0.36 [b]	0.94 ± 0.28	2.07 ± 0.45 [a]
	output H^+ (mmol/h)	19.2 ± 2.03	3.35 ± 2.11 [b]	18.51 ± 1.92	4.43 ± 1.05 [b]	24.2 ± 2.60	3.98 ± 1.25 [b]

O – omeprazole; OA – omeprazole, amoxicillin; OAT – omeprazole, amoxicillin, tinidazole
1 – before treatment; 2 – end of a two-week treatment
[a] $p<0.01$, [b] $p<0.001$ vs before treatment

Table 2. **Salivary bicarbonate; joint analysis of three therapeutic groups (mean ± S.E.)**

	basal conditions		stimulation with catheter		stimulation with catheter and pentagastrin	
	1	2	1	2	1	2
concentration (μmol/ml)	9.47 ± 0.8	7.44 ± 0.51 [b]	8.28 ± 0.66	6.97 ± 0.48 [a]	8.95 ± 0.68	6.39 ± 0.38 [c]
output (μmol/min)	3.44 ± 0.39	2.55 ± 0.28 [a]	8.05 ± 1.65	5.17 ± 0.65 [a]	8.58 ± 1.53	4.80 ± 0.51 [b]

1 – before treatment; 2 – end of a two-week treatment
[a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$ vs before treatment

The mechanism by which gastric acid could modify salivary bicarbonate secretion is unclear. There are no data available for the regulation done through the gastro-salivary reflex. However, the obtained results may be easily explained through the oesophago-salivary reflex. Previous findings have provided evidence that the decrease in the oesophageal pH to 2.1 causes a two-fold increase in the salivary bicarbonate secretion, while at pH 1.3 the increase is even three-fold [1]. It is unknown at what pH the stimulation of salivary bicarbonate secretion is totally extinct; this is likely to occur at pH 2.2 [5]. A reduction in the oesophageal pH related to the gastric reflux is sporadic in healthy subjects, while relatively frequent in patients with duodenal ulcer [15]. Since erosive oesophagitis is a result of gastroesophageal reflux, duodenal ulcer patients with coexistent erosive oesophagitis were not recruited. Unfortunately, the intraoesophageal pH was not monitored and thus it is unknown whether the changes in the intraoesophageal pH took place during saliva collection. As at the end of the treatment gastric pH measured under basal conditions was higher than 3.5 in all therapeutic groups, stimulation of the salivary glands via the oesophago-salivary reflex must have been completely inhibited. Therefore, it can be assumed that in our study only sufficiently low gastric pH before treatment could affect salivary bicarbonate and thus explain their higher values at that time.

Assuming that oesophago-salivary reflex is involved in the regulation of salivary bicarbonate secretion, it seemed rational to collect saliva not only under basal conditions but also during a gastric secretory test with pentagastrin. The intragastric catheter inserted transnasally disturbs the function of the lower oesophageal sphincter and thus promotes gastric reflux to the oesophagus [16,17], while pentagastrin by increasing gastric acid secretion makes stimulation of pH sensitive receptors in the lower oesophagus more efficient during gastroesophageal reflux [5]. The present findings indicate that the catheter had no significant effect on the concentration and output of bicarbonate assessed at the end of the treatment, compared to the baseline, in any of the three therapeutic groups. Since the catheter in the oesophagus could trigger local defence mechanisms by increasing the secretion of oesophageal submucosal glands, similar in composition to that of the oral salivary glands [18-21], the stimulation of oesophageal chemoreceptors by the gastric refluxate could be then attenuated. The higher output of bicarbonate in subjects tested with than without catheter may be explained by the enhanced salivary secretion resulting from catheter stimulation of mechanoreceptors located in the throat and oesophagus.

In the second hour of the gastric secretory test patients with a constantly maintained catheter additionally received pentagastrin as a secretory stimulus. The effect of decreased intragastric pH on the concentration and output of salivary bicarbonate was however manifested in the way similar to that observed in patients without additional stimuli. This is likely related to the fact that pentagastrin, apart from the stimulatory effect on gastric secretion, increases the secretion of the salivary glands as well as submucosal glands of the oesophagus [6,22], and causes an increase in the lower oesophageal sphincter tension, protecting against gastroesophageal reflux [23].

It cannot be excluded that the lack of significant differences

in the concentration and output of bicarbonate before and at the end of the treatment in some of the subgroups analysed separately, may be associated with a small number of patients and a relatively wide dispersion of the results. Nevertheless, in joint analysis of all groups, the concentration and output of salivary bicarbonate as well as the secretion of gastric acid, decreased significantly at the end of the treatment both under basal conditions and when stimulation with a catheter or catheter and pentagastrin was used. No correlation found between the secretion of gastric acid and salivary bicarbonate could be related to the fact that the regulation tested may be responsible only in part for total salivary bicarbonate secretion.

According to some authors, the increase in salivary bicarbonate output, associated with a decrease in intraoesophageal pH, is only related to the increase in salivary secretion and results from the induction of oesophago-salivary reflex [1]. The present findings suggest that in duodenal ulcer patients not only the output but also the concentration of bicarbonate is the subject to changes, and thus mechanisms other than oesophago-salivary reflex can also be involved in the regulation described above. The limitation of our study is a lack of observation documenting salivary bicarbonate secretion after the cessation of antisecretory therapy. However, on the basis of our former studies, we can speculate that it will increase reaching or even exceeding the pre-treatment values [24].

The results of our study provide evidence for the partial involvement of hydrogen ions of gastric origin in the regulation of salivary bicarbonate secretion in duodenal ulcer patients and suggest that disturbed regulation of salivary bicarbonate in some of these patients may predispose to the development of oesophagitis.

Acknowledgments

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Secretory function of the esophageal mucosa in opossum: the role of cholinergic, peptidergic and histaminergic pathways

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Abstract

Purpose: Esophageal histology in the opossum reveals numerous submucosal mucous glands closely resembling those commonly found in humans. The aim of the study was to examine the secretion of these glands using the commonest secretagogues.

Material and methods: The esophageal lumen in 5 male opossums was continuously perfused with saline during sixteen consecutive 5 min perfusion periods. After four initial equilibrating periods, each animal was injected (s.c.) with pentagastrin (6 μg/kg), bethanechol chloride (100 μg/kg) or histamine dihydrochloride (0.5 mg/kg). All sixteen (5 min) perfusates were assayed for protein by Lowry, mucin by PAS and viscosity using a cone/plate digital viscometer. Results were expressed as mean ± SE. Statistical analysis was performed using paired Student's t-test.

Results: Administration of bethanechol resulted in a significant increase in esophageal mucin release from 2.4 ± 0.4 to 8.0 ± 1.2 μg/cm²/min ($p < 0.01$); enhancement of protein output from 8.9 ± 2.0 to 20.4 ± 2.9 μg/cm²/min ($p < 0.01$) and potentiation of specific viscosity from 7.5 ± 0.6 to 14.4 ± 0.8 ($p < 0.01$). Pentagastrin-induced release of mucin reached the maximal value of 5.5 ± 0.7 μg/cm²/min ($p < 0.01$), protein output increased to 20.0 ± 2.7 ($p < 0.01$) and viscosity expanded to 11.7 ± 0.9 ($p < 0.05$). Histamine evoked an increase in mucin release to 3.9 ± 0.4 μg/cm²/min ($p < 0.01$), protein output to 24.1 ± 3.3 μg/cm²/min ($p < 0.01$) and viscosity to 12.8 ± 1.1 ($p < 0.05$).

Conclusions: The significant influence of cholinergic, histaminergic and peptidergic stimulation on physical and chemical properties of the esophageal secretion provides evidence for the role of these pathways in the pathophysiology of the esophageal mucosa.

Key words: esophageal secretion, bethanechol, pentagastrin, histamine.

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Introduction

Gastroesophageal reflux disease usually develops either when excessive gastroesophageal reflux cannot be compensated by normal protective mechanisms or when the reflux remains within normal limits but protective mechanisms are severely compromised. The gastric content refluxed into the esophagus can damage the mucosa through the presence of hydrochloric acid, pepsin, bile salts, and pancreatic enzymes. Since the refluxate pH is most frequently acidic, the major injuries factors are hydrochloric acid and pepsin. Esophageal mucosa, due to the anatomical proximity to the deleterious gastric milieu, developed effective mechanisms preventing its damage by aggressive factors. The esophageal mucosa in the cat, opossum and man, although covered by nonkeratinized squamous epithelium, contains numerous submucosal mucous glands [1-4]. The secretion of these glands, although histochemically proven to be rich in mucins, has never been explored in detail. By using an esophageal perfusion catheter specially designed for testing the esophageal secretory function in vivo [5,6], we were able to study the physical and chemical characteristics of the esophageal secretion in the opossum. Knownig that acetylcholine, histamine and gastrin are the commonest secretagogues governing the secretory function within the gastrointestinal mucosa, we explored their influence on the major components of esophageal secretion.

Material and methods

Preparation of animals

Five opossums (3.5 kg, Didelphis virginiana) were used in the study according to a protocol approved by Animal Research Committee. After overnight fasting opossums were anesthetized with ketamine (20 mg/kg i.m.) and infused with sodium pentobarbital (30 mg/kg/h i.v.) throughout the entire experimental procedure.

Esophageal perfusion catheter

Esophageal perfusion in opossums was performed using a specially designed 6-channeled catheter equipped with two balloons (balloons spaced 10 cm apart, and were 20 mm in length) as described previously [5]. Briefly, to ensure an excellent recovery rate of esophageal perfusate, an optimal diameter of the inflated lower balloon was 25 mm (inflated with 8 ml of air) whereas the optimal diameter of the upper balloon was 23 mm (inflated with 7 ml of air).

Catheter channels were designated as follows: the first and largest diameter (3 mm) channel was utilised for aspiration of esophageal secretion elaborated during perfusion. The second channel (1.5 mm) served for gastric aspiration to determine any loss of esophageal perfusate into the gastric lumen from the measurement of [^{14}C] polyethylene glycol. The third and fourth channels (1 mm) were connected directly with the lower and the upper balloons, respectively, to control their pressure and volume. The fifth channel was designed to aspirate saliva and esophageal secretion potentially collecting within the esophageal lumen above the upper balloon. The sixth channel was used as an infusion port for esophageal perfusion.

Esophageal perfusion model

The location of the lower esophageal sphincter was identified through an initial esophageal motility recording using a standard perfusion catheter in a pilot study based on similar size and weight of animals. Therefore, we could identify the optimal location for the subsequent placement of our perfusion catheter. Based on motility, the esophageal perfusion catheter was inserted into the esophagus with the proximal balloon located 14 cm and distal balloon 24 cm from incisors. Such placement of the catheter compartmentalized a 10 cm segment of esophagus extending from below the upper esophageal sphincter to just above the lower esophageal sphincter. This segment of the esophagus, comprising most of its length, was sealed between two balloons and exposed to perfusing solutions during the experimental procedure. In all animals, the placement of the esophageal catheter was performed with the same technique so that the same areas of the esophageal mucosa were exposed to perfusate.

Esophageal perfusion procedure

Esophageal perfusion in each opossum was performed using NaCl (0.15 M). [^{14}C] polyethylene glycol was used as a non-absorbable marker of the esophageal volume. The experimental procedure involved sixteen 5 min periods (80 min). In each 5 min period, fresh perfusion solution was infused into the esophageal lumen and circulated with the flow rate of 10 ml/min. The selection of the perfusion rate was based on preliminary experiments indicating that this rate would not cause distention of the esophagus or tissue damage from shear force. After 5 minutes, the entire volume of perfusate was aspirated, secured for analyses, and replaced with the next solution.

After four initial 5 min periods, when equilibration of esophageal secretory function was obtained, an injection (s.c.) of saline (control) or secretagogue was implemented. Each secretagogue was injected (s.c.) in a separate experimental setting allowing animals to fully recover from the previous procedure; a 7-day wash-up period was applied. Bethanechol chloride (Merck), pentagastrin (Ayerst) and histamine hydrochloride (Sigma) were administered (s.c.) in a dose of 100 μg/kg, 6 μg/kg and 0.5 mg/kg, respectively.

Measurements

The evaluation of mucin in esophageal perfusate was based on the method developed by Mantle et al. [7] and protein on the method developed by Lowry et al. [8]. The viscosity of freshly recovered esophageal perfusates was measured using a cone/plate digital viscometer as described previously [9]. Results are for the lowest shear rate (2.25 sec^{-1}) at CP-40 cone, applying the most physiological shear stress to the samples, and were expressed in specific viscosity units.

Data analysis

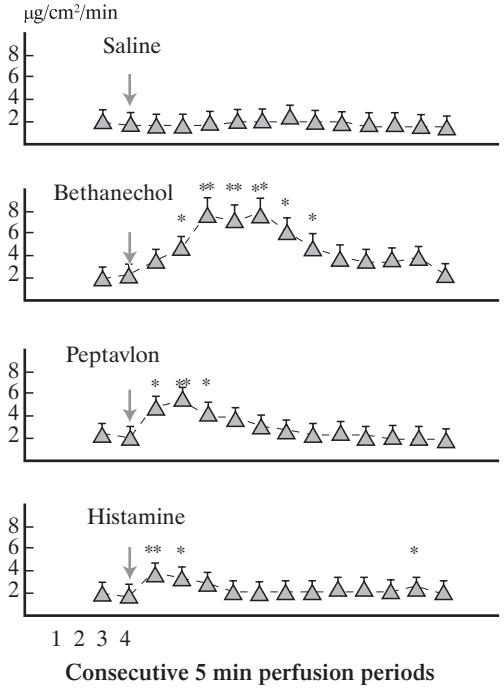
In order to eliminate potential contamination of esophageal perfusate with salivary proteins adsorbed to the esophageal mucosa, we discarded the first two 5 min esophageal perfusate samples from the current analysis. As it can be clearly seen from the control figures, the rate of luminal release of esophageal secretion components reached a plateau during the third and fourth perfusion periods. Therefore, in all samples statistical analysis was performed to compare data obtained after injection of secretagogue (between the fifth and sixteenth perfusion periods) with that recorded in the fourth perfusion period (the last before injection of secretagogues). Results are expressed as mean ± SE. Statistical analysis was performed using paired Students t-test.

Results

Esophageal mucin release

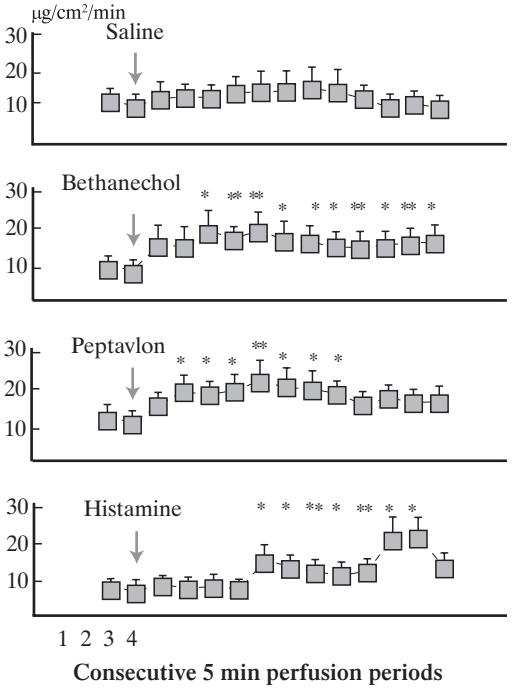
The basal rate of esophageal luminal mucin release was 1.9 ± 0.4 μg/cm^2/min and remained steady throughout the entire control esophageal perfusion procedure with saline (*Fig. 1*). Administration of bethanechol resulted in a significant increase in esophageal mucin release in a step-wise fashion reaching its maximum in 15 minutes from the moment of injection (8.0 ± 1.2 μg/cm^2/min; $p < 0.01$). A significant effect of bethanechol on esophageal mucin release lasted 30 minutes, although at the end of the experimental procedure (60 min), mucin release was still higher than that observed in control animals. The maximal secretory effect of pentagastrin was achieved 5 min faster than that after bethanechol and was also statistically significant (5.5 ± 0.7 vs 2.2 ± 0.4 μg/cm^2/min; $p < 0.01$). The effect of pentagastrin lasted only 15 minutes,

Figure 1. **Effect of bethanechol, peptavlon and histamine on the luminal release of mucin**



* $p < 0.05$; ** $p < 0.01$ as compared to the corresponding values recorded during 4th perfusion period. Arrow indicates the time of injection (s.c.) of the investigated agent

Figure 2. **Effect of bethanechol, peptavlon and histamine on the luminal release of protein**



* $p < 0.05$; ** $p < 0.01$ as compared to the corresponding values revealed during 4th perfusion period. Arrow indicates the time of injection (s.c.) of the investigated agent

although some sustained elevation of mucin release was observed for 30 minutes. Administration of histamine resulted in the fastest secretory response of all secretagogues since it occurred within the first 5 minutes (3.9 ± 0.4 vs 2.0 ± 0.4 μg/cm²/min; $p < 0.01$). This effect, however, was short-lived, although some potentiation of mucin release also took place near the end of perfusion procedure.

Esophageal protein output

Esophageal perfusion with saline resulted in a continuous release of esophageal protein into the perfusate (*Fig. 2*), reaching steady-state phenomenon (12.4 ± 2.8 μg/cm²/min) within the third and fourth perfusion periods in control experiments. Administration of bethanechol resulted in a profound and significant increase in the luminal protein release, reaching its maximum within fifteen minutes of the stimulation (20.4 ± 2.9 vs 8.9 ± 2.0 μg/cm²/min; $p < 0.05$). This stimulatory effect of bethanechol remained significant to the end of the experimental procedure. Pentagastrin also evoked a well sustained stimulatory effect on the luminal release of protein reaching a significantly higher value of 20.0 ± 2.7 μg/cm²/min ($p < 0.05$) after 10 minutes from its injection. The effect of pentagastrin lasted 40 minutes. The effect of histamine on protein release was evidently biphasic with the first peak during the ninth perfusion period and the second at the end of the perfusion procedure.

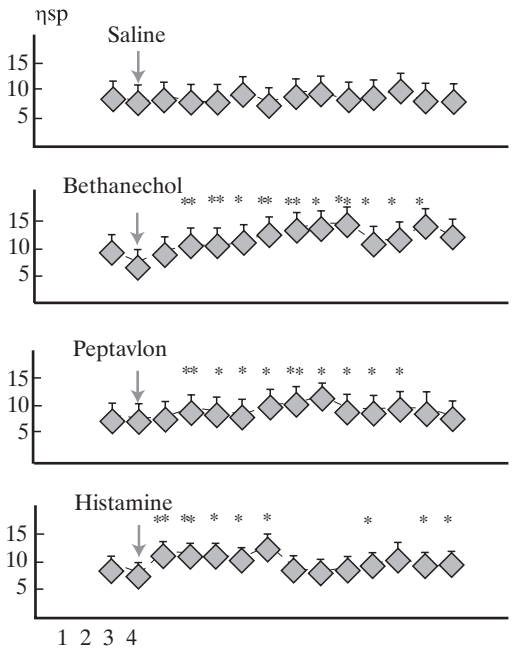
Viscosity of esophageal secretion

The viscosity of the esophageal secretion (*Fig. 3*), measured at the lowest shear rate (2.25 sec^{-1}), mimicking the naturally existing physical forces in vivo, revealed that its value remained unchanged throughout the entire perfusion procedure in control experiments. Administration of bethanechol resulted in a step-wise potentiation of esophageal secretion viscosity reaching its maximal value of 14.4 ± 0.8 vs 7.5 ± 0.6 ($p < 0.01$) during the twelfth consecutive perfusion period and was maintained almost throughout the entire perfusion procedure. Also, pentagastrin evoked a long-lasting stimulatory effect on esophageal secretion viscosity, reaching its maximum during the eleventh consecutive perfusion period (11.7 ± 0.9 vs 7.8 ± 0.7; $p < 0.05$). Even histamine administration resulted in a significant and long-lasting effect on esophageal secretion viscosity, reaching its maximum of 12.8 ± 1.1 vs 8.0 ± 0.5 ($p < 0.05$) at the ninth consecutive perfusion period.

Discussion

The integrity of the alimentary tract mucosa depends upon equilibrium between aggressive factors and protective mechanisms. Although the role of gastric acid and pepsin in the pathophysiology of the esophageal mucosa has been well explored, the role of esophageal mucosal secretion as an

Figure 3. **Effect of bethanechol, peptavlon and histamine on the viscosity of esophageal perfusate**



* $p < 0.05$; ** $p < 0.01$ as compared to the corresponding values measured during 4th perfusion period. Arrow indicates the time of injection (s.c.) of the investigated agent

essential protective factor against damaging agents has not been studied in detail. It has been previously shown that the cat and human esophageal mucosa, sharing similar mucosal and submucosal morphology, have a secretory potential to continuous luminal release of mucin [5,10], epidermal growth factor [6], PGE_2 [11], and bicarbonate [12]. Since numerous submucosal mucous glands are also histologically proven in the opossum [3,4], we have studied their secretion after stimulation with the main secretagogues governing the secretory function of the gastrointestinal tract.

In previous study pentagastrin administered i.v. in a dose of 2 μg/kg/h significantly enhanced gastric mucus secretion in humans [13]. Also histamine (0.05 mg/kg s.c.) was reported to increase gastric mucin output in both controls and patients with gastritis [14]. Furthermore, it has been shown that the biosynthesis of canine gastric mucin was significantly enhanced in response to carbamylcholine, gastrin and histamine [15]. This indicates that gastric mucin synthesis and secretion remain under the significant impact of cholinergic, histaminergic and peptidergic pathways. Our present data suggest that the same pathways exist within esophageal mucosal compartment in opossum.

We have found significant enhancement of esophageal protein release under the impact of all applied stimuli. The origin of this protein remains unknown. We may assume that submucosal mucous glands are the most likely source of these components. Some proteins may have originated from the serum (predominantly albumin) due to an increase in permeability of the mucosa after secretagogues [16], however the contribution of this source of protein may be smaller than that from submucosal glands.

The reason for biphasic effect of secretagogues on protein and mucin release in the fasted state may be explained by coexistent periodic secretion related to the motility patterns. It has been shown that during fasting, the propagation of the migrating motor complex is associated with an increase of secretion in the stomach, pancreas, and intestines [17]. Is it the case also in the esophagus, so far there are no data.

Despite the relatively large volume (10 ml) of the circulating for 5 minutes solution of saline, we were able to record quite high specific viscosity of the esophageal perfusate. Although the mucin is the strongest contributing factor to the viscosity of esophageal perfusate, total protein affects its value as well; this was particularly evident after histamine administration. Considering the fact that viscosity of the esophageal secretion is decisive in the maintenance of the mucus layer on the surface of the epithelium [18], we are convinced that such a layer is able to inhibit hydrogen ion diffusion, and thus contribute significantly to the maintenance of the esophageal mucosal integrity [19].

The results of the present study indicate that esophageal secretion in the opossum remains under the significant influence of cholinergic, peptidergic, and histaminergic pathways and that the strongest secretory response was observed during cholinergic stimulation, followed by pentagastrin and histamine. These data, if confirmed in humans may open new avenues for advanced understanding of the esophageal mucosa pathophysiology. It may also help to design more efficacious treatment for some patients with reflux esophagitis.

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The effect of endothelin-1, endothelin-2 and endothelin-3 in early cerulein-induced acute pancreatitis in rats

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Abstract

Purpose: To assess the effect of endothelins: ET-1, ET-2 and ET-3 on trypsinogen activation, lipase activity and histological changes in the pancreas in early (4 hrs) cerulein acute pancreatitis (AP) in rats.

Material and methods: In 45 Wistar rats with cerulein induced AP (2x40 μg/kg i.p. at 1 hour interval, the effect of endothelins at the dose 2x0.5 or 2x1.0 nmol/kg i.p. was assessed vs untreated AP; 6 healthy rats were control (C). Free active trypsin (FAT), total potential trypsin after activation with enterokinase (TPT), lipase in 12 000 xg supernatants of pancreatic homogenates and the plasma α-amylase were assayed. The %FAT/TPT was an index of trypsinogen activation.

Results: %FAT/TPT increased from 3.0±0.6 in C to 16.2±3.1 in AP ($p < 0.01$). ET-1 decreased this index to 4.8±1.1 after higher dose ($p < 0.01$); the effect of lower dose was insignificant. Attenuating effect of ET-2 was significant: 7.3±1.7 after higher dose ($p < 0.05$) and 6.1±0.9 after lower dose ($p < 0.01$). ET-3 diminished this index to 4.5±1.5 ($p < 0.01$) and to 6.3±2.2 ($p < 0.05$) respectively. Lipase activity in supernatant increased from 4.1±0.6 in C to 6.3±0.7 U/mg protein in untreated AP ($p < 0.05$) and plasma α-amylase from 7.0±0.6 in C to 25.9±4.3 U/ml in AP ($p < 0.001$), without essential changes in treated groups vs untreated AP. Higher doses of endothelins decreased inflammatory cell infiltration score in AP.

Conclusions: The exogenous endothelins, especially ET-2 and ET-3 and to lesser extent ET-1 exerted some protective effect in early, edematous acute pancreatitis by the attenuation of trypsinogen activation and inflammatory cell infiltration in the pancreas.

Key words: cerulein acute pancreatitis, endothelins, trypsin, lipase, α-amylase, histology.

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Introduction

The derangement of pancreatic microcirculation plays an important role in the pathogenesis of acute pancreatitis (AP). The edematous AP is characterized by almost double increase of pancreatic capillary flow (PCF) within 6 hrs, whereas in severe, necrotic form of AP, PCF decreases by half at this time [1]. Early, premature activation of trypsinogen in pancreatic tissue is thought to be a key factor in the pathogenesis of AP [2]. The interrelationships between alterations of PCF and pancreatic zymogen activation have been suggested [3,4].

Endothelin, a 21-residue polypeptide, isolated by Yanagisawa et al. [5] from the culture of porcine epithelial cells has been shown to be the most potent vasoconstrictor known to date. Later, three distinct human endothelins (ET) – related genes were identified and three corresponding familiar polypeptides (ET-1, ET-2 and ET-3) have been synthesized [6]. In the dog pancreas, ET-1 and ET-3 reduced pancreatic blood flow by half, whereas ET-2 only by 20% [7]. All forms of endothelins reduced pancreatic blood flow in healthy rats, however ET-1 was much more effective than ET-2 or ET-3 on a molecular basis [8].

ET-1 is thought to exert its effects by binding to two different receptors, classified as ET_A and ET_B. ET_A receptor has been found to be responsible for vasoconstriction, whereas ET_B was considered as a "vasodilator" receptor, mediating the release of vasodilating factors [9]. Recently, two subtypes of ET_B receptor: ET_{B1}, mediating vasodilatation, and ET_{B2}, contributing to the vasoconstrictor effects, have been identified [10]. In the rat

pancreas ET-1 and ET-3 mRNA and two classes of ET receptors have been found. ET_A receptor had a high affinity for ET-1, but a low affinity for ET-3, and ET_B receptor with equally high affinities for ET-1 and ET-3. No specific receptor for ET-2 was identified in this study [11].

In recent years a broad range of ET-1 receptor antagonists, less or more specific for the receptors ET_A or ET_B have been synthesized, enabling the studies of endothelin-1 action in different severe pathological entities [12-14]. The data on the effects of ET-1 and antagonists of its receptors in AP considerably differs, depending on the disease model, doses and the time of application [15-19]. Kogire et al. [20] found a protective effect of ET-1 infused simultaneously with cerulein on histological changes of the pancreas, whereas selective ETA receptor antagonist, BQ 123 increased pancreatic edema and inflammatory cell infiltration. On the contrary, in the studies of Liu et al. [21,22], the exogenous ET-1 has been shown to reduce pancreatic perfusion and aggravate mild cerulein-induced AP to a severe, necrotic form, whereas BQ 123 attenuated these changes.

The role of ET-2 and ET-3 in acute pancreatitis has not been studied as of yet. If endothelins could attenuate important pathways of early edematous pancreatitis, they could be useful in the prevention of AP escalation. If not, perhaps further studies on different antagonists of endothelin receptors might offer such an advantage.

Therefore, the purpose of the present study was to assess and to compare the effect of different endothelins: ET-1, ET-2 and ET-3 on the activation of trypsinogen, activities of other pancreatic enzymes and histological changes in the early course of edematous acute pancreatitis in rats.

Materials and methods

Animals

The experiments were carried out on 51 male Wistar rats, weighing 240-300 g, housed individually in wire bottomed cages in a room temperature of 21 ± 1°C using a 12 hours light – dark cycle. The animals were given a standard rat chow diet and fasted overnight before the experiment with free access to water. The care was provided in accordance with the current procedures for the care and use of laboratory animals. The protocol has been approved by the local Bioethical Commission.

Induction of acute pancreatitis: Acute cerulein pancreatitis was induced according to the method of Yamaguchi et al. [23]. The rats were injected with cerulein (Sigma Chemical Co., St. Louis, MO, U.S.A.) at a dose of 40 μg/kg of body weight (b.w.) intraperitoneally (i.p.) twice in 1 hour interval. In control rats, only solvent of cerulein (saline solution) was given i.p. In the treated rats, the solution of respective endothelins in NaCl 0.9% was given i.p. twice, simultaneously with cerulein.

Experimental design

Rats were subdivided into 8 groups as follows:

Group I. Control group (C), healthy rats, receiving only saline solution (NaCl 0.9%) i.p. at time 0 and 1 hour later (n=6).

Group II. Rats with cerulein-induced acute pancreatitis (AP) untreated. The solution of cerulein in equivalent volume of saline was given i.p. at 0 time and 1 hour later (n=8).

Group III. Rats with cerulein-induced AP, treated with ET-1 at a dose of 0.5 nmol/kg b.w i.p. twice, in 1 hr interval, simultaneously with cerulein (n=6).

Group IV. Rats with cerulein-induced AP treated with ET-1, at a dose of 1.0 nmol/kg b.w. i.p. twice, as in the group III (n=6).

Group V. Rats with cerulein AP treated with ET-2, at a dose of 0.5 nmol/kg b.w. i.p. twice, in 1 hr interval, simultaneously with cerulein (n=7).

Group VI. Rats with cerulein AP treated with ET-2, at a dose of 1.0 nmol/kg b.w. i.p. twice, as in the group III (n=6).

Group VII. Rats with cerulein AP treated with ET-3, at a dose of 0.5 nmol/kg b.w. i.p. twice, in 1 hr interval, simultaneously with cerulein (n=6).

Group VIII. Rats with cerulein AP treated ET-3, at a dose of 1.0 nmol/kg b.w. i.p. twice, as in the group III (n=6).

The volume of NaCl 0.9% as a solvent was equilibrated in all groups to 2x2 ml/kg b.w.

Preparation of pancreatic homogenate and the plasma: Four hours after the first cerulein injection (or saline in C group) a general anesthesia was induced with intraperitoneal ketamine at a dose of 40 mg/kg b.w., supported by pentobarbital at a dose of 20 mg/kg b.w. The blood samples was taken to the heparinized syringe by cardiac puncture and the rats were sacrificed by decapitation. The pancreases were quickly excised, freed from the peripancreatic tissues and weighed. For light microscopy, representative specimens of the pancreas were fixed and the sections were stained with hematoxylin and eosin.

The remaining portion of the pancreas was processed according to Yamaguchi et al. [23], meaning it was homogenized in ice-cold four volumes of 50 mmol/l Tris-HCl buffer (pH 8.0), containing nonorganic detergent Triton X-100, 0.5% v/v during 1 min by 3 full up-and down strokes using a motor driven glass-Teflon homogenizer (Thomas Scientific, New Jersey, U.S.A.) cooled with ice. The resulting homogenate was sonified for 20 seconds in an ice bath using Vibra cell, model VC 50, Sonics and Materials Inc., Danbury, CT, U.S.A. (frequency 20 kHz and amplitude 70). The volumes were then adjusted giving 10% homogenates, placed on ice for 20 min for further extraction of the enzymes, and then centrifuged at 12000 x g for 20 min at 4°C. The supernatants were used for the assays of trypsin activity performed within 6 hrs. The remaining portions of the supernatant were frozen at -80°C, for the assay of lipase activity and protein concentration.

The samples of heparinized blood were centrifuged at 4000 rpm with cooling to 4°C, the resulting plasma was collected and frozen at -80°C for the assay of α-amylase activity.

Biochemical assays

1. Trypsin activity: Free active trypsin (FAT) and total potential trypsin (TPT) in the supernatants of pancreatic homogenates were estimated according to Yamaguchi et al. [23] with this exception that Nα-p-tosyl-L-arginine methyl ester hydrochloride (TAME) 1 mmol/l was used as a substrate and the absorbance of released product was estimated at 247 nm wave length in an automatic spectrophotometer Pye Unicam SP 505 (Cambridge, U.K.) as in our previous studies [24,25].

Table 1. **Free active trypsin (FAT), total potential trypsin (TPT) and the index of trypsinogen activation (% FAT/TPT) in the supernatants of pancreatic homogenates in early cerulein-induced acute pancreatitis (AP) untreated and treated with different endothelins (ET-1, ET-2 and ET-3) vs control group(C) in rats. Means ± S.E.M**

Nº	Group	FAT μg/mg protein	TPT μg/protein	% FAT/TPT
I	Control (C) (n=6)	0.366 ±0.083	11.95 ±1.20	3.0 ±0.6
II	AP untreated (n=8)	1.254 ±0.145	8.70 ±1.50	16.2 ±3.1
III	AP + ET-1 (n=6) 2 x 0.5 nmol/kg	1.670 ±0.260	11.46 ±0.92	15.3 ±2.9
IV	AP + ET-1 (n=6) 2 x 1.0 nmol/kg	0.607 ±0.120	14.17 ±1.66	4.8 ±1.1
V	AP + ET-2 (n=7) 2 x 0.5 nmol/kg	1.102 ±0.189	16.14 ±1.34	7.2 ±1.4
VI	AP + ET-2 (n=6) 2 x 1.0 nmol/kg	1.097 ±0.156	18.11 ±1.52	6.1 ±0.9
VII	AP + ET-3 (n=6) 2 x 0.5 nmol/kg	0.661 ±0.153	16.14 ±1.11	4.5 ±1.5
VIII	AP + ET-3 (n=6) 2 x 1.0 nmol/kg	1.073 ±0.377	17.03 ±0.53	6.3 ±2.2

Important statistical significance of differences between groups:
FAT: I/II $p<0.001$, II/IV $p<0.01$, II/VII $p<0.02$;
TPT: I/II n.s., II/III $p<0.02$, II/IV $p<0.05$, II/V $p<0.001$, II/VI $p<0.01$, II/VII $p<0.001$, III/VIII $p<0.001$;
% FAT/TPT: I/II $p<0.01$, II/IV $p<0.01$, II/V $p<0.05$, II/VI $p<0.01$, II/VII $p<0.001$, II/VIII $p<0.05$, III/IV $p<0.01$

Total potential trypsin (TPT) in the supernatants was estimated after activation of trypsinogen with enterokinase in 1:1 dilution in 50 mmol Tris-HCl buffer, pH 8.0 for 30 min at 37ºC. The freshly prepared working solution of enterokinase contained 2 mg of enzyme/ml of the same buffer [23]. The time of activation proved to be sufficient for maximal activation.

The activity was expressed in μg of trypsin/mg of protein by comparison with the calibration curve of increasing concentrations of bovine trypsin, type I. The % FAT/TPT ratio served as an index of trypsinogen activation [23].

2. Lipase activity in the supernatants of pancreatic homogenates was assayed with tributyrin (1,2,3 – tributylglycerol) as a substrate and with the pehametric method using autotitrator (Radiometer, Copenhagen, Denmark) and 0.2 mol/l NaOH as in our previous studies [24,25].

3. a-Amylase activity in the plasma was assayed with colorimetric method with soluble starch as substrate as in our previous studies [26,27].

All reagents, with the exception of soluble starch were purchased from Sigma Chemicals Co., St Louis, MO, U.S.A.

Histological examination: Ten slides from 5 rats from each group (50 slides per group) stained with hematoxyline and eosine (H&E) were evaluated at a magnification of 200x in light microscopy by an expert pathologist (A. A.), who was not familiar with the experimental code at this time. The edema, inflammatory infiltrate, necrosis and vacuolization were scored from 0 to 3 degrees of severity according to Kyogoku et al. [28]. Generally, the interstitial edema was scored as follows: 0 = absent; 1 = expansion of interlobular septa; 2 = expansion of intralobular septa; 3 = separation of acini. The inflammatory infiltrate: 0 = absent; 1 = less than 20 neutrophils per field; 2 = 20-50 neutrophils; 3 = more than 50 neutrophils per field. Parenchymal necrosis: 0 = absent; 1 = less than approximately 5%; 2 = 5-20%; 3 = more than 20% of the involved area. The vacuolization: 0 = absent; 1 = less than 20% of acinar cells with vacuoles per field; 2 = 20-50%; 3 = more than 50%.

Statistical analysis. The results of biochemical assays are reported as means ± S.E.M. and after performing an F test for the equality of variances, the means were compared using the t-test for unpaired data. Histologic data were expressed as range of the scores and means ± S.E.M. and compared using Mann-Whitney's test for two groups. The differences with $p < 0.05$ were considered statistically significant.

Results

Tab. 1 illustrates the activities of trypsin and the index of trypsinogen activation in the supernatants containing enzymes extracted using organic detergent Triton X-100. FAT in cerulein AP group is markedly higher (about 3 times) than in the control group ($p < 0.001$), whereas a decrease of TPT activity was insignificant. In the groups with AP treated with endothelins, the increase of FAT was attenuated by half only after higher dose of ET-1 ($p < 0.01$) and after lower dose of ET-3 ($p < 0.02$). In all groups with AP treated with endothelins, TPT was higher than in untreated group with AP, but not significantly different in comparison to control group. The degree of trypsinogen activation (%FAT/TPT) in the untreated group was evidently higher (5.4x) than in the control group ($p < 0.01$). It was attenuated 2.25-3.60 times by the treatment with higher dose of ET-1 and with both doses of ET-2 and ET-3. However, lower dose of ET-1 was ineffective in this respect. Nevertheless, the values of %FAT/TPT in treated groups with AP, after effective attenuation of the trypsinogen activation remained 50%-140% higher than in the control group.

Tab. 2 shows the activity of lipase in the supernatant of pancreatic homogenate from untreated AP, which was ca 50% higher than in control group ($p < 0.05$). The effect of the treatment with endothelins on this activity was insignificant, with the exception of some diminishing influence of higher dose of ET-3 ($p < 0.02$). The plasma α-amylase activity was 3.7 times elevated in cerulein AP ($p < 0.001$) and none treatment affected significantly this activity.

The histological scores of edema, inflammatory cell infiltration, necrosis and vacuolization were significantly increased in the groups with AP, supporting the development of cerulein-induced pancreatitis. The mean score of edema was slightly

Table 2. **Lipase activity in the supernatants of pancreatic homogenates and plasma α-amylase in cerulein-induced acute pancreatitis (AP) untreated and treated with different endothelins (ET-1, ET-2 and ET-3) vs control group (C) in rats. Means ± S.E.M. are reported**

Nº.	Group	Lipase U/mg protein	α-Amylase U/ml
I	Control (C) (n=6)	4.11 ±0.58	7.0 ±0.63
II	AP untreated (n =8)	6.34 ±0.69	25.9 ±4.28
III	AP + ET-1 2 x 0.5 nmol/kg (n=6)	7.67 ±1.36	19.2 ±4.66
IV	AP + ET-1 2 x 1.0 nmol/kg (n=6)	5.53 ±0.84	18.7 ±3.28
V	AP + ET-2 2 x 0.5 nmol/kg (n=7)	6.68 ±0.77	27.2 ±2.98
VI	AP + ET-2 2 x 1.0 nmol/kg (n=6)	7.00 ±0.87	21.6 ±5.54
VII	AP + ET-3 2 x 0.5 nmol/kg (n =6)	4.53 ±0.86	26.4 ±2.62
VIII	AP + ET-3 2 x 1.0 nmol/kg (n=6)	3.87 ±0.42	29.8 ±3.63

Important statistical significance of differences:
Lipase: I/II $p<0.05$, II/VIII $p<0.02$;
α-Amylase: I/II $p<0.001$

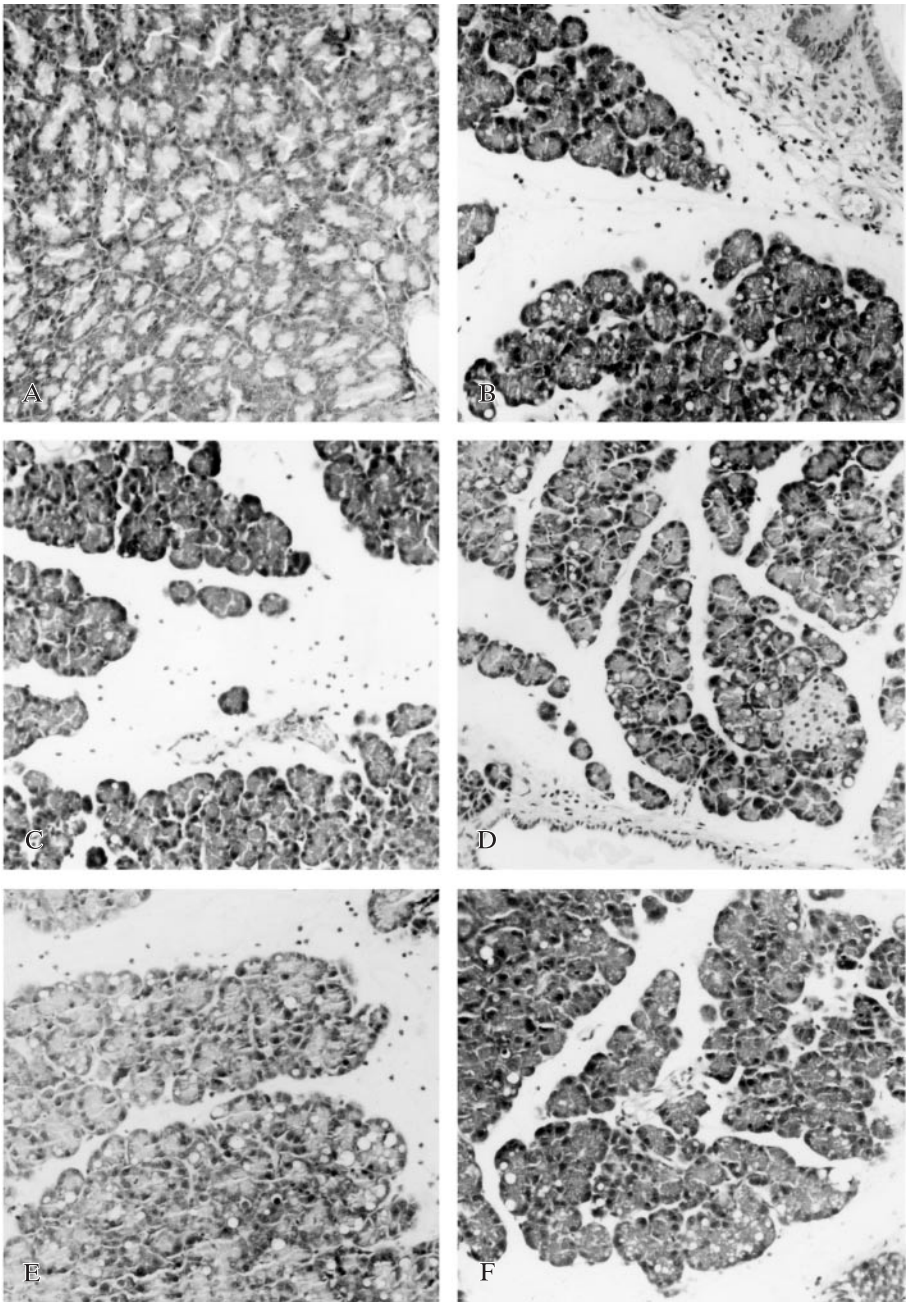
Table 3. **Morphologic changes in the pancreas in early caerulein-induced AP in rats in untread group with AP and in groups treated with endothelins (ET-1, ET-2 and ET-3)#**

No	Group	Edema	PMN infiltration	Necrosis	Vacuolization
I	Control (C) (n=6)	0-1 (0.12±0.03)	0-1 (0.06±0.02)	0-0 (0.00±0.00)	0-1 0.08±0.03
II	AP untreated (n=8)	1-3 (2.04±0.07)	0-3 (1.61±0.08)	0-2 (0.58±0.06)	1-3 (1.88±0.08)
III	AP + ET-1 2x0.5 nmol/kg (n=6)	2-3 (2.62±0.05)	0-3 (1.47±0.09)	0-2 (0.57±0.06)	1-3 (1.96±0.07)
IV	AP + ET-1 2x1.0 nmol/kg (n=6)	1-3 (2.12±0.08)	0-2 (1.04±0.05)	0-2 (0.54±0.06)	1-3 (1.92±0.08)
V	AP + ET-2 2x0.5 nmol/kg (n=7)	1-3 (2.61±0.05)	1-3 (1.59±0.05)	0-2 (0.81±0.07)	1-3 (2.30±0.07)
VI	AP + ET-2 2x1.0 nmol/kg (n=6)	0-3 (2.30±0.08)	0-3 (1.29±0.08)	0-2 (0.63±0.06)	1-3 (1.89±0.08)
VII	AP + ET-3 2x0.5 nmol/kg (n=6)	1-3 (2.32±0.07)	0-3 (1.55±0.08)	0-2 (0.82±0.06)	1-3 (2.18±0.08)
VIII	AP + ET-3 2x1.0 nmol/kg (n=6)	1-3 (2.04±0.08)	0-3 (1.36±0.06)	0-2 (0.70±0.06)	1-3 (1.94±0.08)

Values are expressed as range of scores, with means ±S.E.M. in parentheses.
Important statistical significance of differences:
Edema: I/II $p<0.001$, II/III,V $p<0.001$, II/VI,VII $p<0.01$; III/IV $p<0.001$;
PMN infiltration: I/II $p<0.001$, II/IV $p<0.001$, II/VI $p<0.02$, II/VIII $p<0.05$;
Necrosis: I/II $p<0.001$, II/V $p<0.05$, II/VII $p<0.02$;
Vacuolization: I/II $p<0.001$, II/V $p<0.01$, II/VII $p<0.05$

increased in the group treated with lower doses of ET-1 and ET-2, and even less elevated after higher dose of ET-2 and lower dose of ET-3. The infiltration with polymorphonuclear (PMN) cells was markedly decreased after higher dose of ET-1 and to less extent after higher dose of ET-2 and ET-3. The mean scores of necrosis and vacuolization were comparable in untreated and treated groups with AP, with the exception of the groups after lower dose of ET-2 and ET-3, where some slight elevation of this score was observed (*Tab. 3*). The representative features of histological changes in AP groups in comparison to control group are depicted on *Fig. 1.*

Figure 1. **The representative histological features of pancreatic tissue in early (4 hrs) cerulein-induced acute pancreatitis (AP) untreated and treated with different endothelins. (*A*). Normal appearance of the rat pancreas. (B). Acute cerulein-induced (2 x 40 μg/kg b.w. i.p. at 1 hour interval), edematous pancreatitis. (*C*). Cerulein-induced AP treated with lower dose (2 x 0.5 nmol/kg b.w. i.p.) of endothelin-1, given simultaneously with cerulein. (*D*). Cerulein-induced AP treated with higher dose (2 x 1.0 nmol/kg b.w. i.p.) of endothelin-1. (*E*). Cerulein-induced AP treated with higher dose (2 x 1.0 nmol/kg b.w. i.p.) of endothelin-2. (*F*). Cerulein induced AP treated with higher dose (2 x 1.0 nmol/kg b.w. i.p.) of endothelin-3. Magnification x160. Staining: Hematoxyline and eosine (H&E)**



Discussion

Our results indicate the significant, 2.25-3.60 times attenuation of augmented trypsinogen activation in the pancreatic tissue of the rats with early (4 hrs) cerulein induced pancreatitis (AP) after treatment with endothelins: ET-1, ET-2, and ET-3 at a dose of 1 nmol/kg b.w., and ET-2 or ET-3 at a dose of 0.5 nmol/kg b.w., given twice introperitoneally at 1 hour interval simultaneously with cerulein. The treatment with endothelins generally did not affect the elevation of pancreatic lipase and plasma α-amylase activities with the exception of higher dose of ET-3, which attenuated to some degree the increase of lipase activity. Parallelly, the higher doses of endothelins studied (especially ET-1) decreased a PMN infiltration of pancreatic tissue in

histological examination. The scores of necrosis and vacuolization were not diminished by endothelins, and even they were slightly increased after lower doses of ET-2 and ET-3. Similarly, the mean scores of edema were slightly increased after lower doses of ET-1, ET-2 and ET-3 and after higher dose of ET-2. Consequently, our results suggest that the endothelins at chosen doses, given at the beginning of cerulein-induced AP could be effective in the attenuation of trypsinogen activation and inflammatory reaction in the early course of edematous pancreatitis. The effect of ET-1 on trypsinogen activation at higher dose was evidently more expressed than insignificant effect of lower dose. On the contrary, similar effects of both doses of ET-2 and ET-3, could suggest that the maximal effect could be achieved even with lower doses of these endothelins.

We have chosen the dosage of endothelins basing on the classical study of their effect on pressor responses in anesthetized rats. In the response to intravenous bolus injection (1 nmol/kg) of ET-1, ET-2 and ET-3, late pressor effect developed 10-20 min after the injection and lasted >1 hour. The mean time required for blood pressure 50% recovery to base-line levels was 90 min for ET-1, 120 min for ET-2 and 50 min for ET-3 [6]. For the prolongation of endothelin action we repeated the bolus after 1 hour, therefore the total higher dose amounted 2 nmol/kg b.w. within 4 hours. To disclose eventual dose response effect, we used also twice lower dosage 0.5 nmol/kg given twice at 1 hour interval. This dose was intermediate between low dose of ET-1 (0.1 nmol/kg) and high dose (1 nmol/kg) used by Filep et al [13] in the study of vascular permeability. This permeability in the pancreas was not affected significantly by ET-1 treatment in their study.

Cerulein-induced pancreatitis is a well established, experimental model of acute interstitial, edematous pancreatitis. It has been shown to involve the interaction of supramaximally stimulating doses of the cholecystokinin analog (cerulein) with low-affinity pancreatic acinar cell cholecystokinin (CCK)–A receptors. The consecutive co-localization of lysosomal cathepsin B with digestive enzyme zymogens in cytoplasmic vacuoles enables intracellular trypsinogen activation, thought to be a "trigger mechanism" of acute pancreatitis [29]. Simultaneous blockade of apical secretion is a prerequisite to bass-lateral output of partially activated zymogens into the interstitial space with a consecutive release of cytokines and vasoactive mediators, causing chemoattraction of inflammatory cells and activation of vascular endothelium [4]. In last years, the role of neutrophils in the intrapancreatic trypsin activation by NADPH oxidase (hydrogen peroxide and superoxide) in cerulein-induced pancreatitis in rodents has been documented [30].

In fact, in our study, the attenuation of trypsinogen activation after higher doses of endothelins was associated with decreased score of inflammatory cell infiltration. Nevertheless, lower doses of ET-2 and ET-3 did not decrease the score of inflammatory infiltration and edema, despite of attenuation of trypsinogen activation and even slight increase of necrosis and vacuolization scores was noted. Moreover, lower dosage of ET-1 affected neither trypsinogen activation nor PMN infiltration or necrosis and vacuolization scores, and even slightly increased the edema score. It could suggest that not all histological aspects of local inflammatory reaction depends on trypsinogen activation. The possible explanation could be independent activation of intracellular trypsinogen and nuclear factor-κB (NF-κB) in cerulein-induced pancreatitis, which was found to be required for the production of chemokines by pancreatic acinar cells, initiating the inflammatory cascade [31].

The mechanism of attenuating effect of endothelins on the trypsinogen activation in early, cerulein-induced AP is not easy to explain. In human acute pancreatitis, the level of endothelin-1 was found to be elevated [32]. It is not known whether it is a component of defensive mechanism or a mediator of injuring effects of AP on other organs. The only known effect of endothelins in healthy pancreas is vasoconstriction, with consequent reducing the pancreatic blood flow, the most prominent after ET-1 on molecular basis [7,8]. Two types of endothelin receptors: ET_A with high affinity for ET-1 and 175 times lower affinity for ET-3 and ET_B, with equally high affinities for ET-1 and ET-3 in the rat pancreatic acini have been found, but endothelins neither stimulated the enzyme secretion nor do they alter intracellular Ca^{+2} or cAMP [11]. Therefore the direct role of endothelins in the physiology of pancreas remains to be elucidated.

Cerulein-induced acute pancreatitis is a mild self-limited form of the disease. However, after additional stress it could progress into severe necro-hemorrhagic form, probably by decreases of pancreatic microperfusion [28]. The early microcirculatory changes in the pancreas with acute cerulein-induced pancreatitis include the increase of pancreatic capillary blood flow, increase permeability of the endothelium and accumulation of extravasated fluid in the perilobular space, which were aggravated after additional cold stress. The permeability changes are followed by a decrease in flow velocity and early delayed leukocyte adherence in the pancreatic microcirculation [33]. The most important difference between mild edematous pancreatitis and severe, necrotic form is evident in the early, 6 hrs period of observation. Pancreatic capillary flow in edematous, cerulein-induced AP rapidly increases to 188% of baseline and remains elevated up to 6 hrs of experiment, whereas in severe necrotic AP it decreases to 47% of baseline during 6 hrs. Complete capillary stasis develops in 38% of capillaries in severe AP and is absent in edematous AP [1]. These differences could be crucial for the explanation of controversial role of endothelin-1 and endothelin-1 receptors in AP reported in the literature.

Liu et al. [21] have observed that ET-1 administered at a dose of 250, 500 or 750 pmol/kg b.w. as intraaortal bolus 4 times at 1 hr intervals immediately after the second cerulein (2 x 10 μg/kg intraperitoneally at 1 hr interval) injection, evoking edematous AP, caused a dose dependent increase in pancreatic damage. The scores of vacuolization and necrosis of acinar cells were the most elevated with the highest dose of endothelin-1. Eibl et al. [17] have compared the effect of exogenous endothelin-1 and selective antagonist of it receptor A, LU-135 252 both in edematous, cerulein-induced AP and in severe, bile salt + cerulein AP. LU-135 252 injected i.v. at a dose of 50 mg/kg, 6 hrs after induction of AP decreased capillary permeability by 69% in the pancreas in edematous (cerulein-induced) pancreatitis and by 63% in severe (bile salt + cerulein-induced pancreatitis), where the increase of permeability in the untreated group was higher. Exogenous ET-1 at a dose of 1.25 μg/kg/hr (it means 0.5 nmol/kg/h, because the m.w. of ET-1 = 2492) i.v. during

6 hours, increased capillary permeability by 104% in the sham operated rats, by 40% in the cerulein-induced pancreatitis but did not further augment the extremal increase of permeability in necrotic pancreatitis. The authors suggest that the increase of capillary permeability by ET-1 is an unfavorable effect in edematous AP, which could be counteracted by selective antagonist of ET_A receptor. On the other hand, Kogire et al. [20], in the rat pancreatitis induced by continuous i.v. infusion of supramaximal dose of cerulein (5 μg/kg/hr) for 3.5 hr, found that exogenous endothelin-1 (infused throughout the cerulein infusion at a dose of 100 pmol/kg/hr) exerted a protective effect. It was associated with less advanced inflammatory cell infiltration, acinar cell vacuolization and a decrease in the pancreatic edema index. The potent ET_A receptor antagonist BQ-123 at a dose of 3 mg/kg/h infused concomitantly with cerulein, further augmented pancreatic edema and the extent of inflammatory cell infiltration was greater than with cerulein alone. According to the authors opinion some protective effect of endothelin-1 in their study could be dependent on the secondary increased production of endogenous prostagladins. In fact, endothelin-1 is known to release prostacyclin [34] and a protective effect of prostacyclin analogs in taurocholate pancreatitis has been found in our previous studies [25,27].

It would be reasonable to assume that ET-1 given in properly chosen dose at the beginning of edematous pancreatitis, when pancreatic blood flow is increased, could exert rather beneficial than injuring effect by attenuating trypsinogen activation and inflammatory infiltration in the pancreas. The same could be pertinent also for ET-2 and ET-3, as their potentially suppressing effect on pancreatic microcirculation may be less important than the effect of ET-1. However, it should be kept in mind that the lower dosage of ET-2 and ET-3 increased slightly the necrosis and vacuolization scores, despite attenuation of trypsinogen activation. Therefore, more studies are needed to resolve their action in different models of AP.

Lipase is a recognized factor in the damage to isolated acinar cells in a degree similar to the action of chymotrypsin [35]. The increase of its activity in pancreatic tissue in our study could support its role in the cellular injury in cerulein-induced AP as reflected by the necrosis and vacuolization scores. Its activity was not depressed by the endothelins treatment (with one exception, which we could not explain). It is tempted to assume, that even after attenuation of trypsinogen activation, the inflammatory process in the pancreas could be perpetuated by lipolytic enzymes, with lipase as a representative in present work. This notion could be supported by an increased activity of the plasma α-amylase in the groups with cerulein AP, despite of the application of endothelins.

In summary our results support the positive effect of endothelin-1, -2, and -3, given simultaneously with cerulein in the course of mild, edematous acute pancreatitis in rats, as evidenced by the attenuation of trypsinogen activation and limitation of inflammatory cell infiltration in the pancreatic tissue. Such effects could be useful against progression of the disease into more severe forms and in the prevention of so called "post-ERCP pancreatitis". However, not all biochemical and histological aspects of AP, studied in this work, are favorable influenced by endothelins in chosen doses. Therefore this issue requires further studies on the mechanism of endothelins action in different models of acute pancreatitis.

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Relationship between gastroesophageal reflux disease and myocardial ischemia. Influence of reflux on temporary activity of autonomic nervous system

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Abstract

Purpose: Assessment of the gastroesophageal reflux disease (GERD) influence on myocardial ischemia and autonomic nervous system (ANS) activity.

Material and methods: In 50 patients with angiographically confirmed ischemic heart disease (IHD) in I–III CCS class, simultaneous 24-hour ECG and esophageal pH-metry monitoring was performed. We assessed: (1) GERD occurrence in patients with IHD, (2) influence of pathological reflux (PR) on myocardial ischemia – number and total duration of ST depression episodes in GERD and non-GERD patients, (3) temporary activity of ANS was determined according to the dynamics of spectral HRV (Heart Rate Variability) analysis components (LF, HF, VLF, LF/HF).

Results: 23 patients (46%) fullfilled the GERD criteria. Patients with GERD had significantly higher number of ST depression episodes (4.13 vs 2.85, $p = 0.013$) as well as longer total duration of ischemia (64.73 vs 35.2 min, $p = 0.034$). Spectral HRV analysis showed the significant decrease of LF/HF ratio ($p < 0.035$), which indicates the sympathovagal balance shift towards the parasympathetic system caused by PR.

Conclusions:

1. GERD is frequent condition in patients with angiographically confirmed IHD. Coexistence of GERD may predispose to the myocardial ischemia.
2. Gastroesophageal reflux may cause the shift of sympathovagal balance towards its parasympathetic component. This mechanism may induce esophago-cardiac reflex, leading to diminished myocardial perfusion.

Key words: ischemic heart disease, gastroesophageal reflux disease, activity of authonomic nervous system, spectral HRV analysis.

Abbreviations: GERD – gastroesophageal reflux disease, ANS – autonomic nervous system, IHD – ischemic heart disease, CCS – Canadian Cardiovascular Society, PR – pathological reflux, TIB – total ischemic burden, LF – low frequency, HF – high frequency, VLF – very low frequency, LH/HF – temporary activity of ANS, TP – total power, PTCA – percutaneous transluminal coronary angioplasty, CABG – coronary artery by-pass graft.

Introduction

Gastroesophageal reflux disease (GERD) is one of the most common diseases of upper part of digestive tract. Symptoms of GERD may be typical (heartburn) or atypical: non-cardiac chest pain (NCCP), cough, asthma symptoms, laryngitis [1,2]. The incidence of GERD is high (30-50%) among patients with ischemic heart disease (IHD) confirmed in angiography [3-5]. Results of numerous studies show mutual influence of both conditions on each other. Nitrates and calcium channel blockers, by reduction of the lower esophageal sphincter tension and relaxation of intramural esophageal muscles, may cause the increase of acidic content regurgitation from stomach to esophagus and may diminish the esophageal clearance. On the other hand, reflux may also increase the myocardial ischemia [3-5]. In experimental model such correlation was confirmed by Chauhan et al., who showed significant reduction of flow in left anterior descendent artery after the esophagus perfusion with acid. This phenomenon was observed both in the group of patients with X syndrome and in patients with atherosclerosis of

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coronary arteries, however it was absent in patients after heart transplantation (denervated heart). These results led to the conclusion, that reduction of myocardial perfusion is caused by nervous reflex, and the phenomenon was called "linked angina" [6,7]. Existence of esophago-cardiac reflex was confirmed in Bortolotti's studies. Inflation of a balloon in esophagus had significant influence on mean RR intervals in ECG recorded before and after the esophagus irritation [8]. The aim of the present study is to assess the influence of gastroesophageal reflux on myocardial ischemia and to investigate the correlation of reflux with components of spectral frequency domain analysis of heart rate variability (HRV). The frequency domain analysis of HRV is mathematical transformation of selected ECG recordings, which enables finding compartments with similiar R-R interval time – Fast Fourier Transform (FFT). These compartments reflect the activity of autonomic nervous system.

Material and methods

The study was conducted in the group of patients with stable IHD in 2nd or 3rd functional class according to CCS (Canadian Cardiovascular Society), with inducible ischemia in non-invasive tests and significant lesions in coronary arteries confirmed in angiography. Patients with urgent indications for coronary arteries revascularization (PTCA or CABG) were excluded from further investigation. At inclusion the intensity of anginal symptoms was analyzed (II or III CCS class), symptoms of GERD were not analyzed. Only patients who did not take drugs reducing the hydrochloric acid production for at least 2 weeks were included into the study. Drug therapy of IHD remained unchanged (neither the types of drugs nor their doses) for a month before the inclusion as well as during the study. In 50 patients (M-42; F-8; mean age 55.9, 37-74) simultaneous 24-hour ECG and pH-metry monitoring was performed.

Esophageal pH-metry:

Esophageal pH was measured with antimon electrode (Syntetics, Sweden) and epidermal reference electrode (Ag/AgCl). In each case the electrode was calibrated, and placed in the esophagus, 5 cm above the manometrically localized lower esophageal sphincter. Polygram software was used to analyze pH (PW-version 2.04 Esophogram-version 2.01).

The following parameters were evaluated in esophageal pH monitoring [9]:

1. Time percentage of esophageal pH lower than 4 during 24 hours (FT – fractional time).

2. Total time of pH lower than 4 (TT pH<4 – total time)

3. A number of pathological refluxes (PR – pathological reflux – drop of esophageal pH<4 lasting more than 5 minutes).

24-hour ECG monitoring: In Holter ECG monitoring ST depression >1 mm was considered significant. ECG monitoring was performed using the Reynolds Medical Pathfinder 700. The following variables of ECG monitoring were assessed:

1. The number of ST segment depression episodes (ST dep);

2. The total time of ST segment depression during 24 hour monitoring (TIB – total ischemic burden).

Spectral HRV analysis was performed only in those patients, in whom >95% of R waves were identified as of sinus origin. After the initial analysis, selected ECG recordings underwent the computerized Fast Fourier Transform (FFT). Analysis of short-term recording was selected in order to evaluate the temporary activity of ANS. The following parameters were assessed [10]:

LF – low frequency [ms^2] range 0.5-0.05 Hz: component of sympathetic nervous system activity; HF – high frequency [ms^2] range 0.15-0.5 Hz: component of parasympathetic nervous system activity; VLF – very low frequency [ms^2] range <0.05 Hz – component expressing the activity of renin – angiotensin system and thermoregulation; LF/HF – temporary activity of ANS (sympathovagal balance); TP – total power [ms^2].

Influence of pathological refluxes (PR) on temporary activity of ANS was evaluated by assessment of dynamic changes in spectral HRV analysis parameters in 3 consecutive compartments:

1 compartment (-1): 10 min before the reflux appearance (control compartment);

2 compartment (0): first 10 minutes of reflux;

3 compartment (+1): 10 following minutes.

Spectral HRV analysis parameters were assessed and changes of the evaluated parameters were analyzed as the results of ANS activity alterations due to PR.

Protocol of the study has been approved by Bioethic Commitee of the Medical University of Bialystok.

Statistical analysis:

Statistical analysis was performed using the SPSS package, release 8.0.0 pl. The data are reported as maximum, minimum, mean and standard deviation (SD). Nonparametric Mann-Whitney U test was used when the data were not normally distributed.

The Wilcoxon Matched-Pairs Signed-Ranks test was used for pairs of dependent variables not normally distributed. A result was considered statistically significant when the P value was <0.05.

Results

Tab. 1 lists major clinical and angiographic data. In coronarography a lesion was considered significant when coronary artery lumen was narrowed by at least 70%, with accompanying symptoms of myocardial ischemia. The results of the 24-hour simultaneous Holter and esophageal pH monitoring are shown in *Tab. 2*. Patients were divided into two groups on the basis of pH-metry results:

- Group 1: GERD (-): 27 (54%) patients with FT<5%; patients not fulfilling the pH criteria for the diagnosis of the gastroesophageal reflux disease.
- Group 2: GERD (+): 23 (46%) patients with FT<5%; patients fulfilling the pH criteria for the diagnosis of the gastroesophageal reflux disease.

There were no differences in mean age, rates of hypertension, diabetes mellitus, past history of myocardial infarction and the severity of angina classified by the CCS between the groups. The severity and localization of coronary lesions, rates of anti-

Table 1. **Clinical and angiographic characteristics of the studied patients**

Study population 50 pts (100%)	N (%)
Arterial hypertension	30 (60)
Diabetes mellitus	13 (26)
Previous myocardial infarction	24 (48)
Angina class II CCS	29 (58)
Angina class III CCS	21 (42)
Beta blockers	45 (90)
Calcium channel blockers	26 (52)
Nitrates	45 (90)
Left anterior descending (LAD)	25 (50)
Circumflex coronary artery (Cx)	25 (50)
Right coronary artery (RCA)	32 (64)
1 vessel disease (1VD)	24 (48)
2 vessel disease (2VD)	20 (40)
3 vessel disease (3VD)	6 (12)

CCS – Canadian Cardiovascular Society, LAD – left anterior descendent coronary artery, Cx – circumflex coronary artery, RCA – right coronary artery, 1 VD – one vessel disease, 2 VD – two vessel disease, 3 VD – three vessel disease

Table 2. **Results of simultaneous monitoring esophageal pH and ECG in the study population**

variable	mean	SD	Minimum	Maximum
FT [%]	10.52	12.24	0.2	45,9
TT [min]	140.42	176.22	2.0	685.0
RP [n]	4.48	6.01	0	29
ST dep [n]	4.36	4.64	0	20
TIB [min]	58.05	77.79	0	452.50

FT [%]: time percentage of pH lower than 4 during 24 hours pH-metry (FT – fractional time); TT [min]: total time of pH<4; RP [n]: number of pathological refluxes; ST dep [n]: number of ST depression episodes; TIB [min]: total time of ST depression during Holter monitoring

Table 3. **Comparison of patients: GERD (-) Group and GERD (+) Group**

	GERD (–) 27 pts (54%)	**GERD (+)** 23 pts (46%)	**p**
Age	56.77 (±10.57)	53.08 (±7.65)	NS
Sex (male)	24 (88.9)	18 (78)	NS
Arterial hypertension	19 (70.4)	11 (47.8)	NS
Diabetes mellitus	2 (25.9)	6 (26.08)	NS
Previous MI	12 (44.4)	12 (52.2)	NS
II CCS	15 (55.5)	14 (60.86)	NS
III CCS	12 (44.5)	9 (39.13)	NS
LAD	16 (59.25)	9 (39.13)	NS
Cx	18 (66.7)	14 (60.86)	NS
RCA	10 (37)	15 (65)	0.09
1VD	14 (51.85)	10 (43.48)	NS
2VD	9 (33.3)	11 (47.8)	NS
3VD	4 (14.8)	2 (8.69)	NS
Beta blockers	24 (88.9)	21 (91.3)	NS
Calcium blockers	16 (59.25)	10 (43.48)	NS
Nitrates	22 (81.5)	23 (100)	NS

CCS – Canadian Cardiovascular Society, LAD – left anterior descendent coronary artery, Cx – circumflex coronary artery, RCA – right coronary artery, 1 VD – one vessel disease, 2 VD – two vessel disease, 3 VD – three vessel disease. Group 1: GERD (-) patients without gastroesophageal reflux disease, Group 2: GERD (+) patients with gastroesophageal reflux disease. (Statystical analysis: Mann – Whitney U Test and Chi^2 test)

Table 4. **The comparison of number of ST depression (ST dep [n]) and total ischemic burden TIB [min] in both, selected according to the results of esophageal pH-metry groups**

	GERD (+)	**GERD** (-)	**p**
ST dep [n]	2.85	4.13	0.013
TIB [min]	35.2	64.73	0.034

St dep – depression of ST segment, TIB – total ischemic burden (Statystical analysis: Mann – Whitney U Test)

anginal drug administration (nitrates, beta blockers, calcium antagonists) were comparable in both groups. The detailed data are shown in *Tab. 3*.

During the 24-hour esophageal pH-monitoring, a total number of 224 pathologic acidic refluxes was recorded in 42 patients (84%). In 23 (46%) patients FT≥5% was shown – these patients fulfilled the GERD criteria (GERD + group). Forty two patients (84%) had 218 ST-segment depression episodes during the 24-hour Holter monitoring. Significantly higher number and longer total duration of ischemic episodes was shown in the GERD (+) group – *Tab. 4*. The impact of reflux on the ANS activity was assessed in cases, when no recurrent refluxes were detected in control compartment (10 min before the reflux) and in 2 following 10-minutes long compartments, starting from the beginning of reflux. The existence of multiple reflux episodes in short time would interfere with the interpretation of collected data. Spectral HRV analysis was performed only when ECG recording was technically good and enabled identification of more than 95% R waves during the monitoring period as of sinus origin. Considering criteria presented above, 85 PR were selected for analysis: mean duration 16.82 min; (5.10-76.90 min); SD 14.90. Spectral HRV analysis was performed in 3 time intervals for each reflux. In every time interval 5 components of spectral HRV analysis were described (*Tab. 5*). *Tab. 6* shows the comparison of all components of spectral HRV analysis in studied time intervals. There were no significant differences in range of VLF i TP in all time intervals. Drop of LF and rise of HF did not reach the statistical significance, but there was a trend in control (-1) and study (0) compartment. Comparison of LF/HF (sympathovagal balance), however, showed statistically significant ($p < 0.036$) decrease of LF/HF in study compartment as compared to the control one. Comparison of LF/HF in compartments (-1) i (0) with compartment (1) did not show any differences.

Discussion

The pH-metric criteria of GERD have been found in almost half of patients (46%) in the studied group of 50 patients with

Table 5. **The values of spectral HRV analysis components in 3 time intervals, (-1) control interval before the reflux, (0) first 10 minutes from the beginning of reflux, (+1) successive 10 minutes following compartment 0**

variable	mean	SD (standard deviation)	minimum	maximum	mediana
VLF(-1)	814.38	1178.03	68.70	8770.84	487.92
LF(-1)	197.57	305.64	11.50	1938.08	101.74
HF(-1)	85.74	144.53	4.18	943.54	38.36
LF/HF(-1)	3.23	2.20	0.49	10.88	2.54
TP(-1)	1106.79	1480.35	108.86	11206.78	707.33
VLF(0)	825.38	1007.37	22.15	6694.10	493.27
LF(0)	183.70	286.05	13.39	1860.85	94.43
HF(0)	86.19	142.14	3.08	964.91	37.20
LF/HF(0)	2.94	1.90	0.17	8.29	678.34
TP(0)	1100.73	1193.94	43.51	6864.01	2.38
VLF(+1)	834.00	1138.00	22.15	7046.88	455.30
LF(+1)	196.19	451.24	13.39	3870.29	97.35
HF(+1)	81.56	180.58	4.00	1600.58	36.74
LF/HF(+1)	3.16	2.42	0.20	10.16	2.31
TP(+1)	1119.20	1416.71	43.51	8066.23	707.78

LF – low frequency [ms^2] range 0.5-0.05 Hz: component of sympathetic nervous system activity; HF – high frequency [ms^2] range 0.15-0.5 Hz: component of parasympathetic nervous system activity; VLF – very low frequency [ms^2] range <0.05 Hz – importance of this component isn't well known, it may express the activity of renin – angiotensin system and thermoregulation; LH/HF – temporary activity of ANS (sympathovagal balance); TP– total power [ms^2]. Data analysis – SPSS

Table 6. **Analysis of spectral HRV components variability in studied time intervals**

	A	B	C	p
VLF(-1)vsVLF(0)	43	42	85	0.7876
VLF(-1)vsVLF(1)	47	38	85	0.8833
VLF(0)vsVLF(1)	44	41	85	0.7342
LF(-1)vsLF(0)	52	33	85	0.0793
LF(-1)vsLF(1)	46	39	85	0.1984
LF(0)vsLF(1)	41	44	85	0.6820
HF(-1)vsHF(0)	36	49	85	0.0816
HF(-1)vsHF(1)	43	42	85	0.8764
HF(0)vsHF(1)	47	38	85	0.1054
LF/HF(-1)vsLF/HF(0)	51	34	85	0.0353*
LF/HF(-1)vsLF/HF(1)	43	41	85	0.7146
LF/HF(0)vsLF/HF(1)	36	49	85	0.2385
TP(-1)vsTP(0)	42	43	85	0.7342
TP(-1)vsTP(1)	46	39	85	0.6454
TP(0)vsTP(1)	43	42	85	0.6917

LF – low frequency [ms^2] range 0.5-0.05 Hz: component of sympathetic nervous system activity; HF – high frequency [ms^2] range 0.15-0.5 Hz: component of parasympathetic nervous system activity; VLF – very low frequency [ms^2] range <0.05 Hz – importance of this component isn't well known, it may express the activity of renin – angiotensin system and thermoregulation; LH/HF – temporary activity of ANS (sympathovagal balance); TP – total power [ms^2]

A: in compared pair first parameter value greater then the second
B: in compared pair first parameter value smaller then the second
C: general number of compared pairs
(Wilcoxon Matched-Pairs Signed-Ranks Test)
* statistically significant

angiographically confirmed ischemic heart disease. Presented results are correspond with the observations of other authors, already quoted in the introduction [3-5]. One of the possible explanations of frequent coexistence of both conditions are common risk factors, both occur more often in advanced age, among smokers and patients with visceral obesity [11]. In 1983 Mellow et al. described the impairment of coronary flow as the result of acidic stimulation of esophagus. In that study coronary flow was evaluated with Doppler transducer located in the left anterior descendent artery [12]. Results of Mellow's study were confirmed by Chauhan et al. [6,7]. In the present study we have shown that spontaneous acidic gastroesophageal reflux may trigger the myocardial ischemia. In GERD (+) patients there were more incidents of ST depression in ECG and their total time was significantly longer than in GERD (-) patients. Lux et al., using the simultaneous ECG and esophageal pH-metry monitoring in 15 patients with atherosclerotic lesions in coronary arteries showed time-dependence of 40% ST depression episodes with esophageal pathology [3]. The mechanism of myocardial ischemia increase by the experimental acidic stimulation of esophagus or spontaneous reflux is probably due to esophagocardiac reflex [6,7].

Frequency domain analysis of HRV is considered to be the best semi-quantitative method of ANS activity evaluation [10,13,14]. There are two methods of analysis: long term recording, used in for global assessment of ANS activity and short term recording, to assess the short term changes of vegetative system components and their balance [10]. According to the results of latter type of spectral HRV analysis, an attempt to explain the impact of PR on temporary activity of vegetative system was undertaken. The results of presented investigation did not show any differences in range of TP and LF components of spectral HRV analysis in studied time intervals. We observed the simultaneous decrease of LF component and increase of HF in study interval. Although neither change alone was statistically significant, this constellation caused statistically significant drop

of LF/HF ($p < 0.036$) before and during PR. Results indicate the short term shift of ANS balance towards its parasympathetic during the pathological gastroesophageal reflux (PR) and showed induction of the esophago-cardiac reflex by PR. Other authors also showed impairment of ANS function in patients with GERD. Blaut et al. found the disturbances of vegetative system in patients with GERD. Those authors, using the method of short term recording showed significant reduction of LF and HF in GERD patients in comparison with healthy individuals [13]. Campo et al. observed reduced activity of sympathetic system in patients with GERD, furthermore, they reported a reverse correlation between the activity of sympathetic system and the total duration of reflux [15]. Interesting results reported Tougas et al. in the group of patients with angina-like chest pain of esophageal origin (acid sensitive patients). Half of the studied patients fulfilled the pH-metric criteria of GERD. The authors showed higher basal heart rate and lower activity of vagal nerve in this group of patients in comparison to control. Stimulation of esophagus with hydrochloric acid caused the significant increase of parasympathetic component HF and decrease of LF/HF during the provocation and return to initial status directly after the infusion [16]. Results of the present investigation show, that PR causes similar alteration of ANS activity, as the one caused by experimental acidic stimulation of esophagus. Patients with esophagus motility disorders may also have the dysfunction of ANS. Pirtniecks et al. found the ANS disorders, mainly of the parasympathetic system, in patients with so called nonspecific esophageal motility disorders [14].

Significance of HRV has been thoroughly investigated in cardiovascular disorders. In 1987 Kleiger et al. showed, that decreased parameters of HRV are the independent risk factor after the myocardial infarction [17]. Dilaveris et al. observed the activation of sympathetic system only in patients with post-exercise myocardial ischemia [18]. The comparison of the quoted results with the results of our study shows that ischemic episode results in different alteration of ANS activity than the one caused by gastroesophageal reflux.

Conclusions

1. Gastroesophageal reflux disease is frequent condition in patients with angiographically confirmed ischemic heart disease. Coexistance of GERD in these patients may predispose to myocardial ischemia.

2. Gastroesophageal reflux may cause the shift of sympathovagal balance towards its parasympathetic component. This mechanism may induce esophago-cardiac reflex, leading to diminished myocardial perfusion.

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Sleep-related breathing disorders in small children with nocturnal acid gastro-oesophageal reflux

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Abstract

Purpose: Coincidence of gastroesophageal reflux disease with obstructive sleep apnea/hypopnea syndrome has been discussed in recent years. Treatment with nasal continuous positive airway pressure (nCPAP) reduces gastroesophageal reflux (GER) in adult patients with obstructive sleep apnea (OSA). Moreover, treatment of gastroesophageal reflux with omeprazole can reduce the severity of obstructive sleep in selected individuals. The aim of the study was to test the hypothesis that gastroesophageal reflux does not influence sleep quality and breathing pattern during sleep in children.

Material and methods: 24 children (14 boys, 10 girls, aged 2 months-3 years) with sleep disturbances indicating GER were studied. Standard polysomnography with parallel recording of 24-h oesophageal monitoring was performed. Apnea/hypopnea index (AHI) in active/REM sleep and quiet/NREM sleep was compared between nocturnal acid GER children (13 children; 7 boys, 6 girls; aged 1.28±0.95y; FRT-18.63±11.83%) and nocturnal acid GER-free controls (11 children; 7 boys, 4 girls; aged 1.64±0.97y; FRT-2.93±2.08%). Exclusion criteria were: 1. laboratory signs of infection (↑OB, ↑CRP, ↑α2-globulin); 2. clinical symptoms of infection in the respiratory tract, the alimentary tract or in the urinary tract.

Results: In children with nocturnal GER higher incidence of obstructive apnea/hypopnea during REM sleep was found: AHI=23.35/h±19.1; (CI 95%11.81-34.89) vs AHI=4.99/h±3.12 in children without nocturnal GER. We found no differences between the groups in saturation <90% time during sleep.

Conclusions: The study confirms coincidence of nocturnal gastroesophageal reflux and sleep-related breathing disorders in children. Higher number of apnea/hypopnea during REM sleep was found in children with nocturnal gastroesophageal reflux.

Key words: sleep apnea, gastroesophageal reflux disease, ALTE, SIDS, polysomnography.

Abbreviations:
ALTE – Apparent Life Threatening Event
CI – confidence interval
FRT – Fractional reflux time
nGER – nocturnal gastroesophageal reflux
OSAHS – obstructive sleep apnea/hypopnea syndrome
SaO2 – arterial oxygen saturation
SIDS – Sudden Infant Death Syndrome
TST – Total Sleep Time.

Introduction

Coincidence of gastroesophageal reflux disease with obstructive sleep apnea/hypopnea syndrome (OSAHS) has been discussed in recent years [1-3]. Obstructive sleep apnea may predispose to nocturnal GER by lowering intrathoracic pressure and increasing arousal and movement frequency. Nasal continuous positive airway pressure (nCPAP) can correct these predisposing factors and may be an effective form of antireflux therapy leading to reduce GER in adult patients with OSAHS [4-6]. Moreover, treatment of gastroesophageal reflux with omeprazole can reduce the severity of obstructive sleep in selected individuals [2].

The role of gastroesophageal reflux in apnea/breathing

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Table 1. **Population characteristics and polysomnographic findings**

	Group I nGER (+)	Group II nGER (-)	p
n	13	11	ns
Age (range, y) (Mean) (CI 95%)	0.08-2.9 1.28 ± 0.95 (0.71-1.85)	0.16-3.08 1.64 ± 0.97 (0.98-2.29)	ns
Male, n (%)	7 (53.8)	7 (63.6)	ns
Body weight >95%	0	0	ns
Total recording time (min)	1324 ± 43	1287 ± 39	ns
Total sleep time (TST) (min)	698 ± 72	591 ± 84	ns
Fractional Reflux Time (FRT) (%) (CI 95%)	18.63 ± 11.83 (11.47-25.78)	2.93 ± 2.08 (1.53-4.33)	p<0.0003
Mean intra-oesophageal pH during sleep (CI 95%)	5.01 ± 0.48 (4.72-5.31)	5.78 ± 0.36 (5.53-6.03)	P<0.0003
Mean intra-oesophageal pH during wakefulness (CI 95%)	5.21 ± 0.57 (4.86-5.55)	5.56 ± 0.32 (5.39-5.82)	ns
Apnea / hypopnea Index AHI (n/h TST) (CI 95%)	23.35 ± 19.1 (11.81-34.89)	4.99 ± 3.12 (2.89-7.09)	p < 0.004
Apnea / hypopnea Index AHI (n/h TST) (active/REM sleep) (CI 95%)	24.84 ± 19.64 (12.97-36.71)	8.54 ± 8.55 (2.79-14.28)	p < 0.018
Apnea / hypopnea Index AHI (n/h TST) (quiet/NREM sleep) (CI 95%)	4.00 ± 3.43 (1.93-6.08)	3.23 ± 2.43 (1.59-4.85)	ns
SaO2<90% (%TST)	3.1 ± 1.4	1.1 ± 0.9	ns

nGER – nocturnal gastroesophageal reflux

difficulties in children has long been studied [7-11]. Despite a large number of studies, a direct causal relationship has not been consistently shown. The aim of the study was to test the hypothesis that gastroesophageal reflux does not influence sleep quality and breathing pattern during sleep in children.

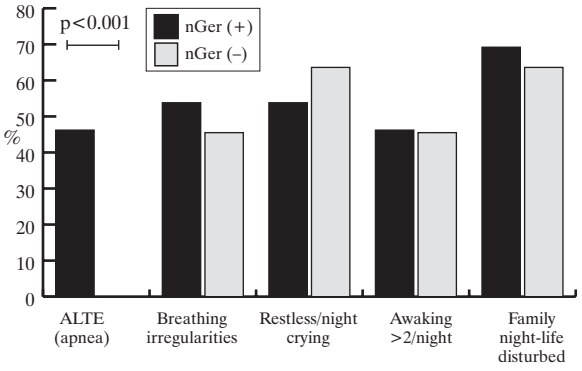
Material and methods

Prospective, nonrandomized study involved 24 children (13 boys, 11 girls) aged 2 months-3 years with chronic sleep disturbances, suspected of nocturnal gastroesophageal reflux (*Tab. 1*, *Fig. 1*). The history, clinical examination, blood tests and polysomnographic study were performed in all the children. Complete polysomnography (Alice 4, Respironics USA) in addition to a 24-hour pH probe (single channel) was performed at the sleep laboratory, in III Department of Pediatrics, Medical University of Białystok. The following parameters were recorded: 2 channels of electroencephalogram (F4A2, F3A1), the bilateral electro-oculogram (LEOG, REOG), chin electromyogram (EMG), nasal and oral airflows detected using a termistor (FLW), chest and abdominal wall movement by respiratory impedance (THO, ABD, Imp), heart rate by electrocardiogram (ECG), arterial oxygen saturation (SaO2) assessed by pulse oximetry with simultaneous recording of the pulse wave form (PLR), body position (Body), actimeter and a digital time-synchronized video recording (*Fig. 2*). All measures were digitized using a commercially available polysomnography system.

Apnea was defined as the cessation of oronasal flow for >5 s and hypopnea was defined as a reduction ≥50% in the oronasal flow for at least 20 seconds associated with a 3% decrease in oxygen saturation. The apnea/hypopnea index (AHI) was defined as the number of obstructive and mixed apnea/hypopnea per hour of total sleep time. Sleep studies were interpreted according to pediatric criteria [12].

The pH probe was introduced through the nose and the tip was sited at 87% of the distance from the nares to the lower

Figure 1. **Clinical symptoms of sleep disturbances**



Sleep disturbances	nGER (+) n=13		nGER(-) n=11	
	n	%	n	%
ALTE (apnea)	6	46,2	0	0
Breathing irregularities	7	53,8	5	45,5
Restless/night crying	7	53,8	7	63,6
Awaking > 2 / night	6	46,2	5	45,5
Family night-life disturbed	9	69,2	7	63,6

Figure 2. **Acid GER event leading to a drop in oxygen saturation (85%) and arousal (↑↓)**

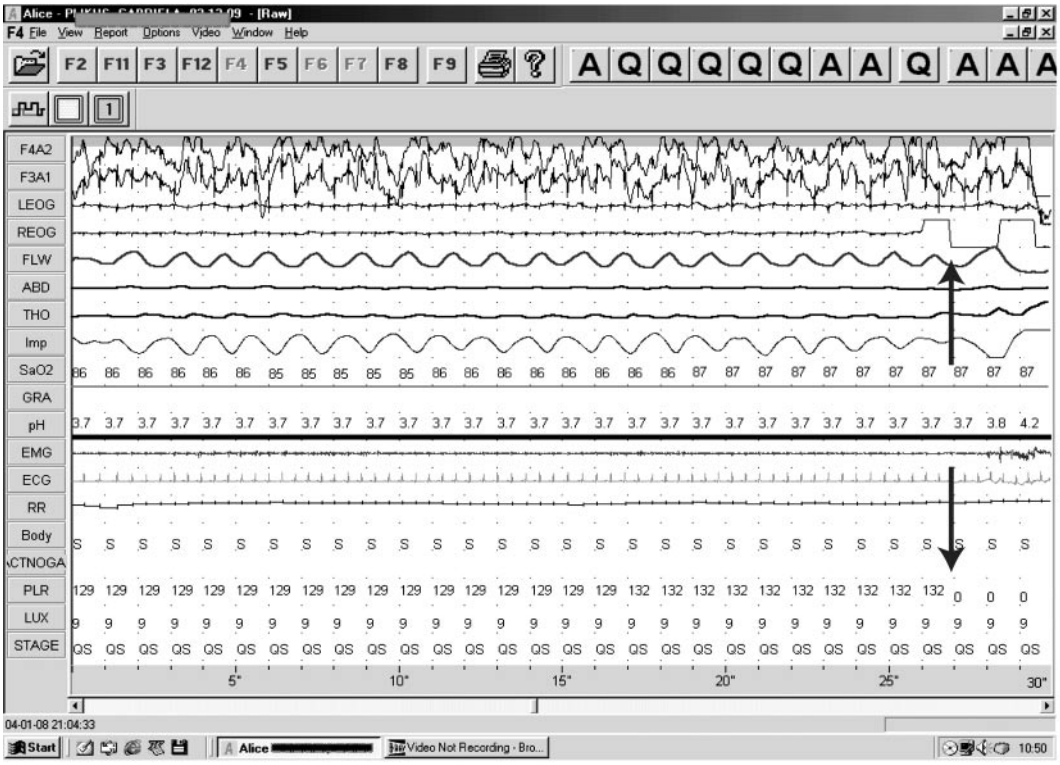
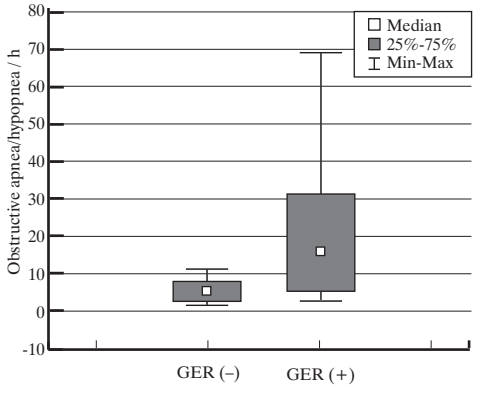


Figure 3. **Sleep-disordered breathing in GER children**



oesophageal sphincter. The reference electrode was attached to the anterior chest wall. Fractional reflux time (FRT), which represents the proportion of the total time of the recording for which the esophageal pH was less than 4.0 was calculated and expressed as a percentage value. When FRT was ≥7% during total sleep time (TST) the diagnosis of nocturnal gastroesophageal reflux was made. None of the study patients took medications at the study time. Exclusion criteria were: 1. laboratory signs of infection (↑OB, ↑CRP, ↑α2-globulin); 2. clinical symptoms of infection in the respiratory tract, in the alimentary tract or in the urinary tract.

Statistical analysis

The Mann-Whitney nonparametric test was used to compare the results in patients with nocturnal gastroesophageal reflux and in control subjects.

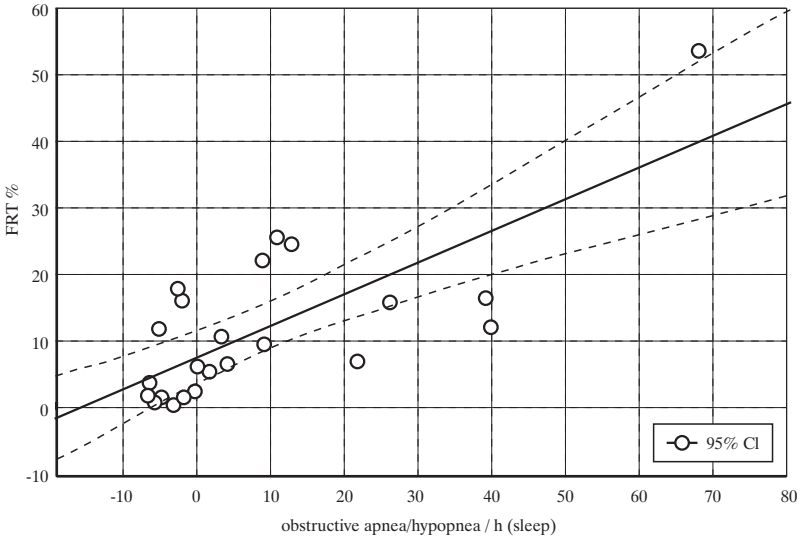
Results

In 13 children (7 boys, 6 girls; aged 1.28 ± 0.95 y) with FRT-18.63 ± 11.83% we recognized nocturnal acid GER (*Tab. 1*). In 11 controls (7 boys, 4 girls; aged 1.64 ± 0.97 y) with FRT-2.93 ± 2.08% we excluded exposure to esophageal acid at night. In children with nocturnal GER the mean intra-oesophageal pH during sleep was higher than in controls. The groups differ significantly in the number of obstructive apnea and hypopnea episodes (*Fig. 3*). This difference referred to the active/REM sleep but not to the quiet/NREM sleep. A strong positive correlation between the intensity of nocturnal acid GER (FRT) and degree of sleep-related breathing disorders (AHI) was found (*Fig. 4*). The total time of saturation < 90% did not differ between the groups.

Discussion

The main finding of this study is that infants and small children with intensive acid nocturnal GER (nGER) present

Figure 4. **Fractional reflux time (FRT) and apnea/hypopnea index (r=0.74482)**



more obstructive apnea and hypopnea during sleep than controls. Obstructive sleep apnea (OSA) is a condition characterized by repetitive, sleep-related obstruction of the upper airway resulting in oxygen desaturation and arousals from sleep. Overnight polysomnography is conducted to assess respiratory, cardiac and neurological abnormalities during sleep and is the optimal standard investigation when sleep apnea is suspected [12,13]. Parallel recording of intraoesophageal pH makes detection of acid gastroesophageal reflux possible. Gastroesophageal reflux with an esophageal pH in the physiological range (pH 5-6.8) is unrecognized by pH-metry and the intraluminal impedance technique is recommended as a diagnostic method [10,14].

The role of gastroesophageal in apnea/breathing difficulties in infants has long been studied, but a direct causal relationship has not been consistently shown [9,13,15]. A high prevalence of GER has been found to be present in adult patients with OSAHS [4-6]. Subjects with OSAHS had more frequent and prolonged reflux episodes than matched control subjects [1]. Nocturnal GER may precipitate symptoms suggestive of OSA, including awaking, nocturnal choking and reduced sleep efficiency. GER symptoms in OSAHS patients are reversed by nasal CPAP treatment, probably by increasing the intrathoracic pressure [4,6]. Moreover, the treatment of GER with omeprazole improves the apnea index (AI) and respiratory disturbances index (RDI) in patients with OSAHS [2].

Obesity which is a common risk factor for OSAHS and nGER in adults, was not found in our study. The nGER group and the control group did not differ in anthropometric parameters. Other predisposing factors, including immaturity of neuro-muscular mechanisms of breathing and swallowing should be considered in this group of age [10,16,17]. The process of inspiration and expiration (the breathing cycle) is precisely linked with the swallowing reflex via the supralaryngeal nerve. It is suggested that OSA is a primary disorder that leads to an abnormality in the swallowing reflex [18]. Patients with OSAHS are likely to exhibit an impaired swallowing reflex, probably due to the perturbed neural and muscular function of the upper airways, resulting in an increased vulnerability to aspiration. During OSA there is an increased respiratory effort by diaphragm, which is transmitted to the lower esophageal sphincter by the phrenoesophageal ligament.

There is evidence that obstructive apneas might cause hypoxia during sleep in infants at risk of sudden infant death syndrome (SIDS). Characteristically petechiae are found on the surface of the lungs and intrathoracic organs such as the thymus at SIDS victims. It has been speculated that these petechiae are due to the highly negative pressure in the thoracic cavity that is generated by the inspiratory effort against an obstructed airway [16]. Polysomnography studies showed more frequent and longer obstructive episodes during sleep in the future SIDS victims [19]. Central apneas were not distinctive. According to the last consensus document of the European Society for the Study and Prevention of Infant Death (ESPID, 2003) digestive disorders are the most frequent problems associated with apparent life threatening events (ALTE) (about 50%) [13]. Gastrooesophageal reflux is one of the main causes identified in ALTE infants (in 28-95% patients) [7,8,20]. When reflux and apnea are associated, the latter is predominantly of the obstructive type. In this study ALTE symptoms were present in 46,2% of nGER patients, but not in the control group.

The present study did not determine a direct relationship between gastroesophageal reflux and sleep-disordered breathing. However, we confirmed their coexistence in small children. Recognition and treatment of GER should help improve the quality of sleep and quality of life in patients having both conditions. It is an important aspect in the management of sleep-related breathing disorders in pediatric patients.

Acknowledgments

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Low serum leptin concentration in vegetarian prepubertal children

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Abstract

Purpose: **Vegetarian diet may play a positive role in reducing risk of several chronic diseases such as diabetes, coronary heart disease and some types of cancer. There are different vegetarian dietary patterns, some of which are nutritionally adequate for children, whereas other may lack some essential nutrients. Leptin, a hormone from adipose tissue plays a key role in the control of body fat stores and energy expenditure. Higher leptin levels were observed in obese subjects and lower in anorectic patients. Recent studies support that diet may be a factor which influences leptin levels. The aim of this study was to investigate serum concentrations of leptin, lipids and apolipoproteins in prepubertal children with two different nutritional habits: vegetarian and omnivorous diet.**

Material and methods: **We examined 22 vegetarians and 13 omnivores in age 2-10 years. Serum leptin concentration was determined by immunoenzyme assay (ELISA) and serum lipids were measured by enzymatic and immunoturbidimetric methods.**

Results: **Average daily dietary energy intake and the percentage of energy from protein, fat and carbohydrates were similar for both groups of children. We observed that in vegetarian diet there is a high rate of fiber nearly twice as high as in omnivorous diet. Vegetarians had lower total cholesterol and HDL- and LDL-cholesterol concentrations than children on traditional mixed diet. There is no significant differences in trigliceride concentration between studied groups. The apolipoproteins levels in vegetarian children were significantly below that of omnivores. The serum concentration of leptin was lower in vegetarians (3.0 ± 1.1 ng/mL) than in nonvegetarians (5.1 ± 2.0 ng/mL) ($p < 0.01$).**

Conclusions: **Our results suggest that vegetarian diet may be accompanied by lower serum leptin concentration. Further studies on large group of children are needed for understanding this problem better.**

Key words: leptin, lipids, vegetarian diet, prepubertal children.

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Introduction

Leptin, a product of the ob gene, is synthesized and secreted mainly from adipose tissue. In humans this hormone regulates feeding behavior, metabolic rate and body energy balance and plays a key role in the control of body fat stores [1,2]. Apart from the function of leptin in the central nervous system on the regulation of energy expenditure it may be one of the hormonal factors, which signal the brain at what time the body is ready for maturation and reproduction [3]. Leptin levels correlate with adiposity, decrease acutely with caloric restriction, and increase with refeeding. Higher circulating levels of leptin were observed in obese subjects and lower in anorectic patients [4-6]. Leptin is important in energy balance, however its role in the metabolism of lipids is still not clear. Recent studies support the concept that other factors such as a diet rich in polyunsaturated fatty acids may influence leptin levels independently on changes in body mass index (BMI) [7].

The principal difference among various vegetarian diets is the extent to which animal products are avoided. Some vegetarian diets provide less fat and fewer calories than typical omnivorous diets and have a higher content of fruits, vegetables, and whole-grain products [8]. There is a wide range of vegetarian dietary patterns, some of which are nutritionally adequate for children, whereas other may lack some essential nutrition. Generally this kind of diet has low caloric density and is very rich in fiber [9].

Table 1. **Serum lipids concentration in prepubertal vegetarian and omnivores children**

	Vegetarian children n=22	Omnivores children n=13	p value
TC (mg/dL)	146.6 ± 23.3	172.4 ± 22.9	p=0.003
HDL-C (mg/dL)	49.3 ± 13.1	60.4 ± 13.9	p=0.027
LDL-C (mg/dL)	80.0 ± 18.5	94.8 ± 15.0	p=0.012
TG (mg/dL)	66.4 ± 22.9	63.6 ± 22.3	NS
Apo A-I (mg/dL)	167.3 ± 23.9	180.2 ± 16.8	p=0.048
Apo B (mg/dL)	69.4 ± 14.1	81.4 ± 18.4	p=0.024

Data are shown as mean ± SD; NS – not significant

However lacto-ovo-vegetarians include dairy and eggs, lacto-vegetarians only dairy, while vegans exclude all animal products. Thus, the choice of vegetarian diet determines the nutritional status and the health of an individual, especially a child.

In the present study we investigated two groups of children with different dietary habits (vegetarians and omnivores) in order to determine the influence of vegetarian lifestyle on leptin and lipids status.

Material and methods

We examined 35 children who had been referred to Pediatric Clinic at the Institute of Mother and Child (Warsaw). Study group consisted of 22 children (11 girls, 11 boys; mean age 5.7 ± 2.9 years) on vegetarian diet. The reasons for the children being seen at Department of Nutrition were dietary consultation. In this group there were 13 children on lacto-ovo-vegetarian diet, 2 on lacto-vegetarian diet and 7 on vegan diet.

As a control group, 13 healthy children (7 girls, 6 boys; mean age 4.5 ± 2.1 years) with normal lipids profile on omnivorous diet were recruited from subjects being under temporary medical supervision (after 2 years of early constipation treatment).

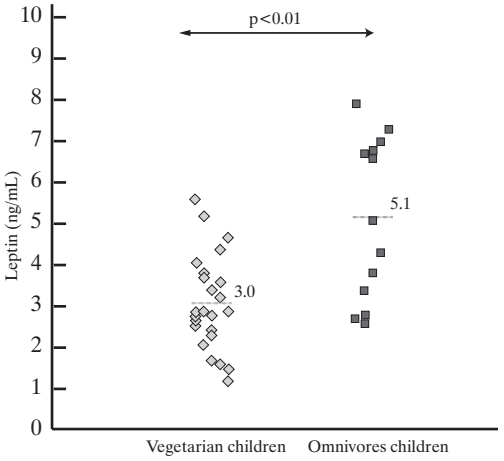
The studies were approved by the institutional review board. Informed consent was obtained from parents of examined children.

We collected main daily diet components and anthropometrical data (weight, height) of all subjects. Dietary constituents were analyzed using nutritional programme „Dietetyk2®" (National Food and Nutrition Institute, Warsaw) and completed with supplementation data. Body mass index was calculated by the formula: body weight (kg) × height (m^2).

Venous blood samples were obtained after a 12-hour overnight fast. Serum was prepared by centrifugation at 1000 x g, at 4^0C. Total cholesterol (TC), high-density lipoprotein HDL (HDL-C), low-density lipoprotein LDL (LDL-C), and triglycerides (TG) concentrations were determined enzymatically with commercially available kits from Bio-Merieux (France) by Cobas Mira analyzer. Serum apolipoproteins A-I (apo A-I) and B (apo B) were measured using immunoturbidimetric metods with kits from Hoffman-La Roche (Switzerland). Serum leptin concentration was determined by immunoenzymeassay (ELISA) using kits from BioVendor Laboratory Medicine, Inc. (Czech Republic).

All data were reported as mean ± standard deviation of the means and compared by Student's t-test. Differences were regarded as statistically significant at $p < 0.05$.

Figure 1. **Serum leptin concentration in vegetarian and omnivores children**



Results

Children consuming vegetarian diet were slightly older (5.7 ± 2.9 years) compared with their omnivorous diet counterparts (4.5 ± 2.1 years). Vegetarian children had slightly higher weight and height, but BMI in both groups was nearly the same: 15.7 ± 1.7 kg/m^2 in vegetarian versus 16.0 ± 1.3 kg/m^2 in omnivores. Average daily dietary energy intake was similar for both groups of children. In vegetarian diet the percentage of energy from protein was: 15.9 ± 3.2; from fat 27.5 ± 6.9; from carbohydrates 56.6 ± 6.9 whereas in the control group it was: 15.6 ± 3.7; 30.2 ± 8.5 and 54.2 ± 9.7, respectively. Vegetarian diets consisted of 20.7 g of fiber and omnivorous – 11.8 g/day ($p < 0.01$).

The results presented in *Tab. 1* indicate that vegetarian children had lower total cholesterol and HDL- and LDL-cholesterol in fractions than meat eaters did. There is no significant difference in TG concentration between the two groups (66.4 ± 22.9 mg/dL in vegetarian and 63.6 ± 22.3 mg/dL in omnivores). The apolipoproteins levels in vegetarian children were significantly below that of nonvegetarians: Apo A-I concentration was 167.3 ± 23.9 mg/dL versus 180.2 ± 16.8 mg/dL ($p < 0.05$) and Apo B was 69.4 ± 14.1 mg/dL versus 81.4 ± 18.4 mg/dL ($p < 0.05$). The mean serum leptin concentration in vegetarian children was significantly lower: 3.0 ± 1.1 ng/mL as compared with the omnivores: 5.1 ± 2.0 ng/mL, ($p < 0.01$) (*Fig. 1*). The

vegetarian children in our study were on different kind of diets, therefore leptin levels for the 13 children on a lacto-ovo vegetarian diet versus the 7 vegans were compared. Mean values of leptin was 3.17 ± 1.1 ng/mL in lacto-ovo vegetarians and slightly lower: 2.9 ± 0.9 ng/mL in vegans (no significant).

Discussion

Winnicki et al. [7] examined leptin levels in two African populations. In one of them the major dietary component was fishes, whereas in the other it was vegetables. Authors stated that diet rich in fish resulted in lower leptin levels in comparison to one in the manner independent on body mass index. Our results in prepubertal children suggest also that vegetarian diet rich in olive oil may be accompanied by lower leptin levels. It seems that diet may influence serum leptin concentrations.

Conclusions

This is the first observation on serum leptin levels in prepubertal children on vegetarian diet and larger studies are needed for better understanding this problem.

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Assessment of selected adhesion molecules and lymphocyte subpopulations in children with IgA nephropathy

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Abstract

Purpose: The aim of the study was to assess the expression of selected adhesion molecules on mononuclear cells of peripheral blood and lymphocyte subpopulations in children with IgA nephropathy (IgAN).

Material and methods: 14 children with IgAN and 20 healthy controls were included in the study. Flow cytometry was used to determine the expression of such adhesion molecules as L selectin (CD62L), VLA-4 integrin (CD49d), intracellular molecule ICAM-1 (CD54) and cytotoxic lymphocyte molecule CTLA-4 (CD152), as well as the lymphocyte antigens: CD3, CD4, CD8, CD19, CD1656 (NK), CD4 and CD8 RO+ and RA+.

Results: The findings revealed that the expression of the adhesion molecules VLA-4 and CTLA-4 did not differ from that of the healthy controls ($p>0.05$). However, the expression of CD62L (L-selectin) was increased ($p<0.05$). The expression of ICAM-1 was reduced, but not significantly, compared to the control group ($p>0.05$). We found a decrease in the expression of NK cells (CD1656) and CD4/CD8 ratio, and an increase in CD8 cells ($p<0.05$). In the group of 9/14 children, with proteinuria over 1.0 g/24 hours, a decreased expression of CD4 was additionally found ($p<0.05$).

Conclusions: The children with IgAN show: 1. Changes in peripheral lymphocyte subpopulations involving an increase in CD8 cells and a decrease in CD1656(NK) cells, a reduction in the CD4/CD8 ratio, and additionally in cases with proteinuria a reduction in CD4 cell count, 2. Increased expression of L-selectin (CD62L) on periferal blood mononuclear cells.

Key words: IgA nephropathy, adhesion molecules, lymphocyte subpopulations.

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Introduction

IgA nephropathy is one of the most common immunological diseases of renal glomeruli in adults. It differs from Schönlein-Henoch purpura, being more common in children in the lack of generalised changes. The final diagnosis of IgA nephropathy is based on renal biopsy and finding immune deposits in glomerular mesangium, which consist of complement and immunoglobulins with a quantitative predominance of immunoglobulin A (IgA). The immunological process leading to the formation of IgA deposits is stimulated mainly by viral and bacterial antigens [1]. These deposits induce a number of reactions with a release of cytokines, growth factors and adhesion molecules, and their interactions enhancing inflammation or repair processes. These processes result in the accumulation of the amorphous matrix in the glomeruli and their sclerosis [2]. Adhesion molecules play a special role in the persistent injury of renal glomeruli in different immunological diseases. The knowledge of the pathogenic role of adhesion molecules in IgA nephropathy can facilitate proper treatment. In the animal model it has been shown that blocking of the adhesion molecules with monoclonal antibodies tempers the course of such diseases as lupus erythematosus, rheumatoid arthritis or Graves–Basedow disease [3,4]. It has been revealed that adhesion molecules, i.e. selectins, integrins and intracellular adhesion molecules [1,7,10], are the sensitive markers of progression in many diseases [3-9]. The earlier studies, especially those using flow cytometry, have demonstrated the role of T-cell associated disturbances of cellular response in the pathogenesis of IgAN. First of all, the abnormalities affect CD4 and CD8 lymphocyte subsets, which has also been

Table 1. **Clinical and laboratory characteristics of examined children IgAN**

patient	age (years)	histhopathologic type (WHO)	erythrocyturia		proteinuria mg/kg b.v./24h	oedema
			micro	macro		
1	14	II	+		6.1	–
2	13	II	+	+	13.4	–
3	6	II	+		2.6	–
4	9	II	+		24.4	–
5	7	II	+	+	2.5	–
6	6	II	+	+	5.4	–
7	8	II	+		28.9	–
8	13	II	+		15.7	–
9	11	III	+	+	11.8	–
10	16	IV	+		9.9	–
11	14	IV	+	+	128.4	+
12	15	V	+	+	61.7	+
13	14	V	+		110.5	+
14	16	V	+	+	82.3	+

confirmed in our own study [11]. In recent years, the role of adhesion molecules in the etiopathogenesis and the course of glomerulonephritis was emphasised. According to literature survey, an increase in adhesion molecules has been shown in neoplastic diseases, in diabetes, hypertension, in patients with impaired renal function, and also in chronic glomerulonephritis [6-9,12]. However, very few data are available on the expression of different adhesion molecules in the subpopulations of peripheral mononuclear cells in the course of IgA nephropathy. Most of the studies are concerned with the assessment of particular adhesion molecules in serum or tissues.

In order to enrich the knowledge on the role of adhesion molecules in IgA nephropathy we decided to assess the expression of L-selectin, VLA-4 integrin, intracellular adhesion molecule ICAM-1 and the receptor of T-lymphocytes – CTLA-4 on the monoclonal cells of peripheral blood.

L-selectin (CD62L, LEC-CAM-1, LAM-1) is expressed on almost all leucocytes. Its role is to facilitate binding of leucocyte glycoprotein to endothelial cells and their passage through the blood capillary wall, and homing the peripheral lymphatic organs by T-lymphocytes. It is the main receptor of homing for circulating unstimulated lymphocytes [10,13,14].

VLA (CD49d/CD29) (very late antigen) is a subunit of integrin $\alpha 4\beta 1$, which possesses 4 α chains and 1 β chain and is distributed on lymphocytes, monocytes, granulocytes, platelets and other cells. It is a receptor localised on the surface of unstimulated lymphocytes, which is bound to the cytoplasmatic protein to transmit the signal to the cell. It has an affinity to fibronectin, which is an extracellular matrix protein, and is responsible for reciprocal adhesion of cells and their adherence to the extracellular matrix. During lymphocyte stimulation by antigens, VLA-4 production is elevated [2,9,10].

The ICAM-1 (CD54) is a vascular adhesion molecule situated mainly on high endothelial cells. It is a ligand for integrins. It is also a receptor which facilitates cell penetration by some viruses. Apart from endothelial cells, ICAM-1 molecules are expressed on lymphocytes, fibroblasts, epithelium and other cells [3,15-19,20].

The CTLA-4 is a co-stimulator, which blocks the lymphocyte proliferation. It is an antigen of cytotoxic T-lymphocytes. The stimulation of CTLA-4 receptors on T-lymphocytes leads to the inhibition of their proliferation, by inducing their apoptosis.

The aim of the study was to assess: 1. The expression of selected adhesion molecules on mononuclear cells of peripheral blood, 2. The subpopulations of peripheral blood lymphocytes in children with IgAN.

Material and methods

The study group consisted of 14 children (♀-3, ♂-11) aged 4-16 years (mean 11.6±3.7), with IgAN (group I). In 9/14 children (subgroup A) constant proteinuria (>1.0 g/24 h) was observed and in 4/14 nephrotic syndrome was found. The control group (C) consisted of 20 healthy children (♀-13 ♂-7) aged 6-17 years (mean 14.0±3.1), without erythrocyturia and proteinuria.

For analysis, 1 ml venous blood samples taken to probes containing K_2EDTA were used. White blood cell counts with smear, lymphocyte subpopulations and some adhesion molecules were determined. Flow cytometry, using a Coultier analyser and monoclonal antibodies (f. Becton Dickinson) was applied to assess peripheral blood lymphocyte subpopulations: CD3, CD4, CD4RO, CD4RA, CD8, CD8RO, CD8RA, CD19, CD56, and adhesion molecules: L selectin (CD62L), VLA-4 integrin (CD49d), intracellular adhesion molecules ICAM-1 (CD54) and cytotoxic lymphocyte molecules CTLA-4 (CD152) on mononuclear cells of blood. The study was performed before treatment of the children.

Statistical analysis was based on a computer program Statistica 6.0, with the t-Students test and Pearson correlation tests. A p value <0.05 was considered statistically significant.

The study was approved by the Ethical Committee, Medical University of Białystok.

Results

Tab. 1 presents clinical and laboratory characteristics the group of children with IgAN. Haevy proteinuria (>10 mg/kg

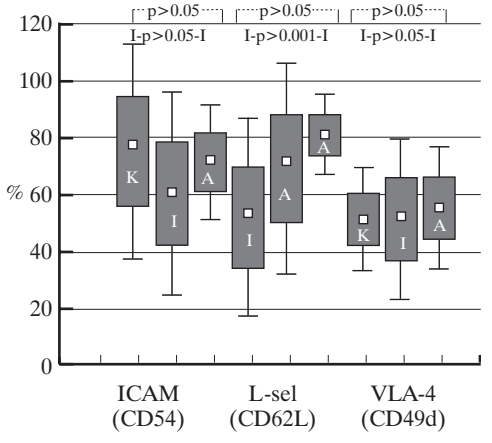
Table 2. **The mean values of lymphocyte subpopulations of peripheral blood in healthy children (C) and children with IgAN (I). Nine children with IgAN groups with heavy proteinuria are presented as subgroup A**

Group C n = 20	white cell count G/l	T CD3 %	T CD4 %	T CD8 %	B CD19 %	NK CD1656 %	CD4/CD8
X	6.20	70.56	43.50	27.26	13.56	12.30	1.58
SD	1.16	6.24	5.62	2.69	3.70	4.30	0.29
min–max	3.5-8.5	59.1-80.8	30.5-50.9	22.2-32.8	6.5-20.0	6.8-18.8	1.11-2.05
Group I n =14							
X	7.25	68.88	35.26	33.43	17.66	8.52	1.15
SD	1.49	8.53	12.10	8.12	9.12	4.13	0.54
min–max	5.2-9.6	51.6-82.8	12.7-52.3	17.8-44.0	6.5-41.0	2.9-14.2	0.3-2.0
p I z K	0.0887	0.8185	0.1203	0.0204	0.1548	0.0037	0.0421
Group A n = 9							
X		71.61	35.60	34.38	15.71	8.57	1.11
SD		7.99	10.29	6.29	7.22	3.85	0.49
min–max		57.0-82.8	20.3-47.4	24.0-44.0	6.5-26.7	3.5-14.2	0.5-1.9
p A z K		0.9132	0.03557	0.0051	0.6862	0.0419	0.0025

Table 3. **The expression the adhesion molecules on lymphocyte of peripheral blood in children with IgAN (I), subgroup IgAN with heavy proteinuria (A), and healthy children (C)**

Group C n = 20	L-selectin (CD62L) %	ICAM-1 (CD54) %	VLA-4 (CD49d) %	CTLA-4 (CD152) %
x	51.91	73.90	51.34	4.35
SD	16.97	18.39	8.79	1.91
min–max	18.1-76.1	16.6-98.1	28.4-66.0	1.6-7.3
Group I n = 14				
x	68.15	59.81	51.30	5.89
SD	18.03	17.39	13.71	3.05
min–max	32.7-87.6	25.7-90.0	16.7-72.7	2.2-12.2
Subgroup A				
x	79.91	65.21	58.30	5.97
SD	23.22	15.32	14.67	3.01
min–max	18.8-98.2	17.1-84.5	16.9-84.6	2.3-13.1

Figure 1. **Comparison of the adhesion molecules on lymphocyte of peripheral blood in children with IgAN (I), IgAN subgroup with heavy proteinuria (A) vs control group (C)**



% – percentage of the lymphocytes with adhesion molecules

b.v./24 h) was observed in 9 children. In 4 children was observed nephrotic proteinuria (> 50 mg/kg b.v./24 h). All the examined children revealed erythrocyturia and periodical haematuria as the predominant clinical symptoms. Renal biopsy showed: II degree of the disease progression in 8 children, III degree in 1 child, IV degree in 2 children, and V degree in 3 children, according to the histhopathological classification proposed WHO [21,22].

All the examined children with IgA nephropathy had normal white cell counts (7.2 ± 1.4 G/l), which did not differ from those of healthy controls ($p > 0.05$). Mean values of peripheral blood lymphocyte subpopulations in the examined groups of children are shown in *Tab. 1*. In group I, including all the children with IgAN, we found a reduced number of CD4 and CD1656 (NK) cells and elevated number of CD8 and CD19 (B lymphocytes), compared to the control group (C). However, the differences were statistically significant only for CD8 and CD1656 cells ($p < 0.05$), compared to healthy children ($p < 0,05$). The CD4/CD8 ratio was significantly depressed ($p < 0.05$). In subgroup A, including the children with IgAN, who had not only erythrocyturia, but also constant proteinuria (more than 1.0 g/24 hours), we additionally revealed a reduced CD4 lymphocyte count ($p < 0.05$).

The mean expressions of the adhesion molecules are presented in *Tab. 2*. In group I, the children with IgAN, showed an increased expression of CD62L cells (L-selectin) in comparison

with the healthy controls (C) ($p < 0.05$). However, the reduced expression of ICAM-1 molecule was not statistically significant. No differences were observed between the expression of integrin VLA-4 and CTLA-4 ($p > 0.05$). *Fig. 1* presents data concerning the expression of adhesion molecules in our patients (group I and subgroup A) vs. healthy controls (C). In subgroup A, including the IgAN children with constant proteinuria, increased expression of L-selectin (CD62L) ($p < 0.01$) and a statistically insignificant increase in integrin VLA-4 expression were found ($p > 0.05$). In the group of patients, no linear correlation was observed between L-selectin and B lymphocytes ($r = -0.183$), NK cells ($r = 0.111$), CD4 lymphocytes ($r = 0.089$), irrespective of the severity of pathological changes in the urine. However, a very weak negative linear correlation was found between the expression of L-selectin and CD4/CD8 ($r = -0.327$).

Discussion

Mononuclear cells play a very important role in the pathogenesis of IgA nephropathy. Their accumulation in the kidneys is conditioned by the adhesive interaction between the cells and the adequate ligands [15,21,23-27]. We assessed the expression of three main types of adhesion molecules and lymphocyte subpopulations in children with IgAN confirmed in the histopathological examination. The findings revealed that the expression of L-selectin on peripheral blood mononuclear cells in children with IgAN was increased and the expression of ICAM-1 was decreased, in comparison with healthy children. However, only the difference in the mean L-selectin expression was statistically significant. The expressions of VLA-4 and CTLA-4 were similar to those observed in the control group. Our findings suggest that L-selectin, which takes part in leucocyte migration and adhesion to endothelium, might play an important role in the pathogenesis of IgAN. Similar results were obtained by Kannel-de March et al. [13], who observed increased proportion of T and B lymphocytes and increased L-selectin expression on T-lymphocytes in their patients. The authors assume that overproduction of CD62 L cells may be related to the increased adhesion of lymphocytes and their homing in the lymphoid tissues in IgAN. Sakatsumi [28] used flow cytometry to assess the antigen expression of T-lymphocytes and monocytes in the urine of patients with different glomerulopathies, including IgAN. They showed the presence of $CD45RO^+$ and $CD62L^-$ T-effector cells, like in renal glomeruli. The authors believe that the analysis of antigen expression on T-lymphocytes in the urine of children with IgAN can facilitate monitoring of the disease activity. The highest expression of these cells was found in patients with heavy proteinuria and impaired renal function. However, $CD62L^+$ lymphocytes were present in the lymphatic follicles, where active cells change into memory cells.

Many reports indicate that vascular adhesins, represented by the intracellular adhesion molecule ICAM-1, also play an important role in the pathogenesis of IgAN [15-18,26,29]. The evidence for that fact is the expression of ICAM-1 on renal cells in patients with IgAN. The study of Arrizabalage [15] suggests that the cellular ICAM-1 in renal tubules and interstitial tissue takes part in leucocyte adhesion. The expression of ICAM-1 in renal tubules is induced by macrophages and interstitial and glomerular T-cells, especially CD8 cells [27]. It has been shown that such cytokins as TNF-α and interleukin 1 together with mononuclear cells induce the regulation of ICAM-1. The authors assume that the cellular expression of ICAM-1 in renal tubules and interstitial tissue may be a marker of tubulointerstitial damage in IgA nephropathy. Ootaka [29] showed that ICAM-1 took part in the induction of proteinuria in IgAN. Tomico [19] found an increased expression of ICAM-1 in the renal glomeruli of patients with IgAN. However the expression did not correlate with the immunological complex, containing IgA. Up to now, ICAM-1 expression on renal tissue has been mainly evaluated [5,15-17,19,26,29]. Few investigators have assessed serum levels of soluble ICAM-1 molecules in IgAN [20,30]. In our study, the expression of ICAM-1 on peripheral blood mononuclear cells was evaluated. A little decrease in ICAM-1 was found in the children with IgAN, compared to healthy controls. A decrease in ICAM-1 expression on peripheral blood mononuclear cells may indicate their utilisation in the immunological process. However, in our examination the decreased expression of ICAM-1 in the study group did not differ statistically significantly from the values in the control group.

In the immunological diseases, integrins are of pathogenic importance [10]. Namie and colleagues [2] estimated the expression of integrins on peripheral lymphocytes and monocytes of patients with IgAN. They found the increased expression of β integrins: VLA-4 and VLA-5. The activation of VLA-4 (fibronectin receptor) on peripheral mononuclear cells indicates its participation in the process of glomerulosclerosis in the course of IgAN. In our investigations the expression of VLA-4 on peripheral lymphocytes and monocytes in patients with IgAN did not differ from that of healthy children. We noted no changes in the expression of the CTLA-4 molecule, either.

The present study revealed a thymus-dependent lymphocyte activation in children with IgAN. This finding was supported by the increased number of CD8 T-cells and decreased CD4/CD8 ratio. Similar results were obtained in our earlier studies in children with Schönlein-Henoch nephropathy [11]. It was also confirmed by other authors, who studied IgAN [23,25,27]. The status of CD4 and CD8 T-lymphocytes is defined by the presence of the receptors $CD54RA^+$ and $CD54RO^+$ on their surface. CD4 or $CD8^+CD45RA^+$ cells are the naive lymphocytes, which have not got into contact with the antigens yet and which after the antigen stimulation change into CD4 or $CD8^+CD45RO^+$ memory lymphocytes [31-33]. In our examinations, antigen expression on lymphocytes did not differ from that observed in the healthy controls. On the other hand, a decreased count of CD1656 cells (NK) was found. One of the properties of NK cells is their participation in the cellular antibody-dependent cytotoxity. In that case a decreased number of NK cells on the peripheral cells in IgAN may indicate their involvement in the immunological process in the kidney [1,11,24,34]. In the urine of 9/14 examined children constant proteinuria (more than 1.0 g/24 hours) was found. A detailed analysis showed an increase in the expression of L-selectin and a reduction in the number of CD4 and CD8 cells – more pronounced than in the children without proteinuria. We noted increased expression of VLA-4 integrin, as well.

Our results confirm the observations of other authors that in IgAN children with concomitant proteinuria, lymphocyte subpopulations and some adhesion molecules, and particularly L-selectin are more involved in the immunological process than in patients with mild course of the disease.

Our results should be further evaluated in a larger study cohorts. There is still an open question: can inhibition of expression of adhesive molecules by neutralizing antibodies, he used as a therapeutic treatment of IgAN.

Conclusions

The children with IgAN show:

• changes in peripheral blood lymphocyte subpopulations involving an increase in CD8 cells and a decrease in CD1656 (NK) cells, a reduction in the CD4/CD8 ratio, and additionally in cases with proteinuria a reduction in CD4 cell count.

• increased expression of L-selectin (CD62L) on peripheral blood mononuclear cells.

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Diagnostic value of birch recombinant allergens (rBet v 1, profilin rBet v 2) in children with pollen-related food allergy

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Abstract

Purpose: Pollen-related food allergy to fresh fruits and vegetables is a well-known clinical phenomenon. Bet v 1, the major birch pollen allergen, has been cloned and shows homologies to various food allergens (e.g. hazelnut, apple, celery, tomato). Allergy to profilin Bet v 2 was also described in 10-15% of patients sensitized to birch pollen. Objective of our work was to assess the diagnostic value of recombinant allergens (rBet v 1, rBet v 2) for diagnosis of children sensitized to birch pollen with associated food allergy.

Material and methods: The investigations were carried out on the group of 14 children aged 4-17 years, with a history of allergic reactions and sensitized to birch pollen with associated food allergy. Skin prick tests were performed with natural foods and commercial aeroallergens (Bencard). Sera-specific IgE antibodies to recombinant and other allergens (Pharmacia Upjohn) were measured with a fluoroimmunoenzymatic essay (UniCAP). Oral food challenge tests were performed to confirm adverse food reactions.

Results: 64% were sensitized to rBet v 1, 14% to rBet v 2, 7% to both of them. 50% of children with allergy to Bet v 1 had also concomitant allergy to other pollens and food allergy to fruits from family Rosaceae. Patients with positive reaction to Bet v 2 represented allergy to vegetables from family Umbelliferae. The most common form of allergic reactions were: allergic rhinitis in 64%, atopic dermatitis in 36%, oral allergy syndrom in 21% of investigated children.

Conclusions: Use of two recombinant allergens permits the diagnosis of birch pollen sensitization in children with food-pollen related allergy and gives the pattern of possible cross-reactivity between pollen and food allergens in children with allergic diseases.

Key words: birch recombinant allergens, cross allergy, children.

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Introduction

Pollen-related food allergy to fresh fruits and vegetables is a well-known clinical phenomenon. It concerns patients sensitized to pollens and suffering from allergic rhinitis and bronchial asthma and developed allergic reactions after ingesting some of plant-origin foods containing allergens from the same family [1,2]. The results of first clinical investigations were published by Eriksson, Onorato, Boccafolgi in the midle of last century [3]. They described association of hypersensitivity to birch pollen with allergy to apple, hazelnuts or coincidence between allergy to grass pollen and celery, carrot and tomato [1-3]. Many immunological data has been obtained using sera provided by birch pollen-sensitive patients with simultaneously occuring allergies to various fruits and vegetables. They explained immunological background and pathogenesis of cross reactivity. The scientifc experiments with new immunological methods (immunoblotting, RAST inhibition) suggested structural similarities in the allergic components responsible for these cross-reactivities [4-6].

Techniques of genetic engineering applied to allergens have enabled the production of highly pure proteins with homogenic structures, identified sequency of peptides and B cell and T cell epitopes (recombinant allergens) and allowed to better understanding of pathogenetic mechanism of cross allergy. In most cases, it concerns the presence of cross-reactive IgE epitopes in pollen and plant-derived food. By using specific

antibody probes, IgE immunoblott inhibition experiments and molecular biology techniques, it could be demonstrated that plant-derived food contains allergens that share epitopes with the major birch pollen allergen, Bet v 1, and profilin Bet v 2, a highly cross-reactive plant panallergen [7-9].

Materials obtained from natural sources often vary in composition and contain many allergens (major and minor), so a reliable separation of the different allergens contained in that material is not always easy to achieve. The epitopes of major allergens (Bet v 1, Mal d 1) show the highest IgE-affinity, but the different patterns of IgE-binding were also observed in individuals. In general, allergen extracts from plant materials contain many glycoproteins with N-linked glycans that bind IgE in a subgroup of patients sensitized to pollen and food [4,5,10].

Last years and new diagnostic methodes gave a better knowledge of allergen structure and the pathomechanism of association between pollen and food allergy. The investigations performed with recombinant and native allergens have confirmed the role of them in diagnosis of cross-reactivity, based on the similarity and homogenicity of protein structure. Most common are also skin tests with recombinant allergens and detection of specific IgE to recombinant polypeptides in the sera of patients. The differences between immune response to recombinant polypeptides can hence be used to decrease or even modulate specific IgE responses in vivo [4,9,11].

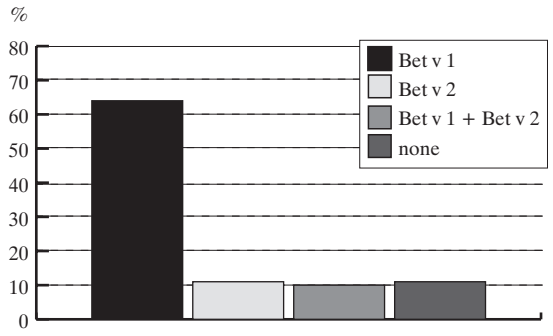
These recent studies concern also allergens of Betula verrucosa. The presence of rBet v 1 (major allergen) and rBet v 2 homologues allergen (profilin) in tree extracts was investigated by IgE immunoblott experiments. Bet v 1 is one of the most important environmental allergens and it shares B cell and T cell epitopes with related allergens present in the pollens of many trees and plant-derived food. Furthermore, more than 95% of birch pollen allergic patients react with Bet v 1, and more than 60% are sensitized exclusively against Bet v 1. Proteins that share common epitopes with Bet v 1, the major birch pollen allergen, occur in pollens of several tree species: apples, stone fruits, celery etc. Molecular analysis has proved the higher amount of IgE-binding epitopes in rBet v 1 allergens versus rBet v 2. Allergy to profilin Bet v 1 is also specific to patients with food allergy to fruits form family Rosaceae [6,9,12].

The first results of investigations with recombinant proteins indicated the necessity of further experiments with plant food and pollen allergens. It is important not only to comprehension of cross allergy, but, first of all, the use of modified recombinant allergens with reduced allergic activity lead to new modalities of specific immunotherapy.

Material and methods

The investigations were carried out on the group of 14 children (10 boys, 4 girls) aged 4-17 years, with a history of clinical allergic reactions to food and pollen allergens. The diagnosis of cross allergy was confirmed on the basis of clinical symptoms and skin prick tests performed as with birch pollen as with fruits and vegetables. In all patients, food allergens were tested in native form by means of a modified skin prick technique (prick by prick tests). To perform tests with aeroallergens (trees and birch pollen, grass, weed pollen), commercial extracts from Bencard Company were used. Negative (saline serum) and positive (9% Codeine) controls were also included. The criterion for positivity of the test was a wheal diameter of 3 mm to tested allergens with a negative glycerosaline control result.

Figure 1. **Positive sIgE to birch pollen recombinant allergens (N=14)**



Sera-specific IgE antibodies to recombinant and other allergens (Pharmacia Upjohn) were measured with a fluoroimmunoenzymatic essay (UniCAP) as detailed by the manufacturer and considered positive if >0.7 kU/l. To confirm adverse food reactions, labial and oral food challenge tests (DBPCFC) were performed, following standards.

Results

All 14 patients who had been included in this study showed positive prick test reactions to birch pollen; sensitization to other pollens was found in the majority of cases. Allergy to birch pollen was also confirmed on the basis of IgE specific results. They were positive in all of investigated patients. 64% of children were sensitized to rBet v 1 (5 of them has IgE antibodies in 3rd class), 14% to rBet v 2, 7% to both of them (major allergen rBet v 1 and profilin rBet v 2) (*Fig. 1*). These, who had reaction neither to Bet v 1 nor to Bet v 2 (14%), presented lower levels of birch IgE in the sera than the others, but had elevated sIgE to artemisia (Artemisia vulgaris) and timothy (Phleum pratense) and suffered from pollen-related food allergy to fruits from family Rosaceae and Rutaceae (citrus). 50% of children with allergy to Bet v 1 had also concomitant allergy to other pollens and food allergy to fruits from family Rosaceae (apple, peach, cherry). But all children with positive reactions to rBet v 2 demonstrated allergy to some fruits and vegetables from family Umbelliferae (celery, carrot, parsley). The most common clinical form of allergic reactions were: allergic rhinitis (64%), atopic dermatitis 36%, bronchial asthma (7%) and multiorgans symptoms as (bronchial asthma, allergic rhinits, atopic dermatitis) observed in 50% of children. 21% of patients suffered from oral allergy syndrom after ingesting of some fruits (apple, peach, citrus, cherry). Analysis of clinical symptoms is presented in *Tab.1*.

All investigated children were underwent labial and oral food challenge with potential harmful food allergens. Positive results were obtained in 50% of children in labial challenge tests

Table 1. **Clinical symptoms in the investigated group of children (N=14)**

Clinical symptoms / Specific IgE to birch	Allergic rhinitis N (%)	Atopic dermatitis N (%)	Bronchial asthma N (%)	Oral allergy syndrome N (%)
Bet v 1 (N=9)	6 (43%)	2 (14%)	-	2 (14%)
Bet v 2 (N=2)	4 (14%)	-	1 (7%)	1 (7%)
Bet v 1 + Bet v 2 (N=1)	1 (7%)	1 (7%)	-	-
None (N=2)	-	2 (14%)	-	-

Table 2. **Positive results of food challenge tests in children with birch-pollen allergy (N=5)**

Patient (initials)	sIgE to birch recombinant allergens			Food allergens (food challenge tests)			
	Bet	Bet v1	Bet v2	Apple	Orange	Peach	Cherry
K.K.	3rd class	3rd class	3rd class	(+)	(-)	(+)	(-)
M.W.	3rd class	3rd class	0 class	(+)	(+)	(+)	(+)
Ł.M.	3rd class	3rd class	0 class	(+)	(+)	(+)	(-)
M.S.	2nd class	0 class	0 class	(+)	(+)	(-)	(-)
M.R.	2nd class	0 class	0 class	(+)	(+)	(-)	(-)

and 36% in oral provocation tests, most often to apple. The results of food challenge tests with comparison to sensitization to recombinat birch allergens are presented in *Tab. 2*.

Discussion

Increasing numbers of clinically relevant cross-reactivities between different pollen and food allergens have been recognized during the last years, due to the progress of molecular biology and immunological studies. Patients with pollen allergy, also to birch, often experience allergic reactions to various fruits, vegetables and nuts [1-3,12]. The major birch pollen allergen is Bet v 1 and the results of investigations showed that more than 95% of birch allergic patients react with Bet v 1 and more than 60% are sensitized exclusively against Bet v 1. This recombinant protein has also homology to other trees of the order Fagales (oak, nut). Although allergic reactions after the ingestion of apple, peach observed in patients with birch-pollen allergy are well described, there are few reports on the homology of epitopes between some fruits, vegetables and second, highly cross-reactive birch pollen allergen – profilin rBet v 2, cloned and expressed in Escherichia coli [9,11].

Patients with food-birch pollen related allergy are more often sensitized to Bet v 1 than to Bet v 2. It has been proved in Rossi et al. investigations carried out on the group of 65 patients presenting rhinoconjunctivitis or asthma and sensitized to tree pollens of trees of the order Fagales. All subjects reacted to at least one of the recombinant birch allergens: 43% to Bet v 1, 30.7% to Bet v 2 and 26% to both of them. Patients with a history of oral allergy syndrome after eating apples were monosensitized and reacted to Bet v 1 in 57%, but subjects allergic to Bet v 2 were polysensitized to other pollens and fruits [13]. Our clinical results indicate also that 64% of children had specific IgE against Bet v 1 and only 14% against Bet v 2. Bet v 1 allergic subjects demonstrated clinical symptoms after ingesting of apple, peach, orange and they with Bet v 2 allergy didn't tolerate celery, parsley.

The clinical statement of pollen-related food allergy is well known, but the clinical suspicion of this coincidence should be confirmed by food challenge tests [14,15]. It is very important, because of the lack of positive correlation between results of skin and food provocation tests. In many cases, skin prick tests with food extracts are not standarized and affected by false negative reactions in patients with clinical symptoms, after eating of some products [14]. The significantly greater importance for evidence of sensitization and correlation with clinical symptoms came from detection of specific antibodies in the serum of patients [16-18]. Serious systemic reactions after eating apples, peach, nuts in birch pollen sensitizied patients, were noticed only in cases with high level of specific IgE antibodies. In contrast, patients with positive prick tests and negative specific antibodies demonstrated only isolated local symptoms (oral allergy syndrom) [13]. Our clinical studies confirm the neccessity of following positive skin prick tests by food challenge tests. Only 5/14 investigated children reacted in provocation tests to apple, orange, peach, cherry. Positive SPT and specific IgE in 2nd and 3rd class were noticed in all subjects.

Patients with birch pollinosis and oral allergy syndrom frequently develop adverse reactions to hazelnuts, what is well known and very common [6,9,12,18]. But in medical history they often include walnuts among causative foods, however, skin prick tests with commercial walnut extracts are almost invariably negative in these subjects, what has been confirmed by recent study performed by Asero et al. The study were carried out on the group of 36 birch-pollen-hypersensitive adult patients reporting OAS after ingestion of nuts. No patients was positive on SPT with fresh walnut and only two with commercial extract, but all patients were positive on SPT with fresh hazelnut. On the basis of these findings, the possible explanation is that walnut does not express any Bet v 1-like allergen and the only way left definitely to diagnose walnut hypersensitivity in these patients

would be the food challenge. In our study in 5/14 children allergy to hazelnut was confirmed; none of them had specific antibodies to walnut [19].

Our and other investigations showed that cross allergy between different fruits (cherry, peach, pear) in patients with allergy to Bet v 1, concerns only those, who have allergy to Bet v 1 (*Tab. 2*) [9,20,21].

The comparative study of Pauli et al., with two recombinant birch pollen allergens, confirmed that sensitization to rBet v 1 is specific for birch and Rosaceae allergies, whereas sensitization to birch profilin, Bet v 2, is encountered in multisensitized subjects and is not always related to Umbelliferae allergy [11]. Immunochemical and molecular biology studies indicate that rBet v 1 contain more IgE binding epitopes than rBet v 2, what explained clinical importance of major Bet v 1 allergen. Rosaceae fruit allergy associated with birch pollinosis is typical to inhabitants in Central and Northern Europe. Rosaceae fruit allergy can occasionally be observed in patients without pollinosis. In those patients, profilin and Bet v 1-related structures are not involved in pathogenesis of food allergy symptoms [21]. This thesis can be counted as controversial but is the first step to further clinical analysis.

Type I allergic symptoms in the oropharyngeal mucosa upon contact with plant-derived food in patients with pollen allergies have been termed oral allergy syndrome (OAS). IgE cross-reactivity between pollen and food allergens represents the molecular basis for this phenomenon. Patients with OAS exhibit a broad variety of symptoms on direct contact of the oral mucosa with plant food as apple, peach, nuts etc., especially during and after the pollen season. This type of food sensitization often concerns people with birch-pollen allergy [2,3,6,12]. IgE immunoblot inhibition experiments demonstrated that plant-derived foods share epitopes with the major birch pollen allergen, rBet v 1 [6]. In the investigated group of children OAS was noticed in 3 of them. Because of the fact that proteins appeared to be strongly degraded in commercial extracts, diagnostic procedures should be based on the native allergens comming from natural sources. In addition, recombinant allergens can be beneficial in diagnostic of OAS [11,13,22].

The actual question remains the value and correlation of skin prick tests, concentration of specific IgE and clinical symptoms in patients with allergy to birch recombinant allergens. In the study of Oster, Pauli, assessing the value of diagnostic tests, total correlation between them was noticed in patients reacted to Bet v 1. In skin prick tests was noticed higher sensitivity with recombinant allergen rBet v 2 I.b., contrary to serum concentration of specific IgE [16].

The recent studies indicate also that only 75% of patients with confirmed IgE cross allergy to Bet v 1 – Mal d 1, demonstrated clinical symptoms after ingesting of apple. Some experiences with recombinant allergens show that these IgE binding with Bet v 1 and its homology in apple Mal d 1 can be clinically irrelevant because of cross-reactive carbohydrate determinants (CCD) [8,23,24]. They are frequently present in patients with adverse reactions to certain foods and responsible for false positive laboratory tests. These observations can explain the absence of specific IgE to birch recombinant allergens (Bet v 1, Bet v 2) obsreved in our two patients with presence of specific IgE to mixture of birch allergens (Bet). Clinically, they demonstrated the severe atopic dermatitis with allergic rhinonjunctivitis and showed positive skin tests to some of inhalant and food allergens (orange, apple, nuts). Food allergy was confirmed on the basis of food challenge tests. Fernandez--Rivas et al. investigations indicate that allergy to Rosaceae fruits (apple, peach) can be observed in patients without a related pollen allergy and profilin and Bet v 1-related structures are not involved in this kind of fruits allergy [21].

Recombinant allergens are not only very important in diagnostic procedures. Some recent progress has led to the production of modified recombinant allergens: the synthesis of recombinant polypeptides corresponding to T epitopes, the production of isoform recombinant allergens with reduced allergenic activity [25,26]. These isoforms can be used as a new tool in specific immunotherapy and reduce the risk of systemic reactions [26].

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The way to cardio-renal protection in non-diabetic chronic nephropathies

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Abstract

Angiotensin II (AII), the main effector of the Renin Angiotensin System (RAS), plays a central role in the hemodynamic and non-hemodynamic mechanisms of chronic renal disease and is currently the main target of interventions aimed to prevent the onset and progression of chronic nephropathies to end stage renal disease (ESRD). In addition to ameliorate glomerular hyperfiltration and size-selectivity, reduce protein traffick and prevent glomerular and tubulo-interstitial toxicity of ultrafiltered proteins, RAS inhibitors also limit the direct nephrotoxic effects of AII. Thus, both ACE inhibitors (ACEi) and AII antagonists (ATA) exert a specific nephroprotective effect in both experimental and human chronic renal disease. This effect is time-dependent and is observed across degrees of renal insufficiency. Forced ACEi or ATA up-titration above doses recommended to control arterial hypertension and combined treatment with both agents allow to optimise A II inhibition and maximize renoprotection. Multifactorial interventions combining RAS inhibition to treatments targeted also to non-RAS mechanisms may even achieve regression of glomerulosclerosis and chronic tubulo-interstitial injury. Studies are needed to assess whether renal damage can be reverted to such a point that renal function may be fully prevented from worsen, and possibly improve. The economic impact of even a partial improvement would be enormous. Moreover, chronic renal insufficiency is an independent risk factor for cardiovascular disease and effective nephroprotection may also decrease the excess cardiovascular morbidity and mortality associated with chronic nephropathies. In patients with renal insufficiency ACEi are even more cardioprotective than in those without, and are well tolerated. Thus, RAS inhibitor therapy should be offered to all renal patients without specific contraindications, including those closer to renal replacement therapy.

Key words: cardio-renal protection, non-diabetic chronic nephropathies.

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The key role of angiotensin II in the pathogenesis and progression of chronic renal disease

Angiotensin II (A II), the main effector of the Renin Angiotensin System (RAS), plays a central role in the hemodynamic and non-hemodynamic mechanisms of chronic renal disease and is currently the main target of interventions aimed to prevent the onset and progression of chronic nephropathies to end stage renal disease (ESRD) [1]. In vivo, AII enhances the vascular tone of both afferent and efferent glomerular arterioles, modulating intraglomerular capillary pressure and glomerular filtration rate. AII exerts its vasoconstrictor effect predominantly on the postglomerular arterioles thereby increasing the glomerular hydraulic pressure and the filtration fraction (glomerular hyperfiltration). High glomerular capillary pressure increases the radius of the pores in the glomerular membrane, thus impairing the size-selective function of the membrane to plasma macromolecules [2]. In isolated perfused kidneys infusion of A II results in a loss of glomerular size-selectivity and proteinuria, an effect that has been attributed not only to the hemodynamic activity of AII [3], but also to its direct effect on the glomerular barrier [4]. Podocytes have a complex cytoskeleton with contractile properties, and there are AII receptors on their surface [5]: these findings have suggested that AII may alter perm-selective

properties of the glomerular barrier by mediating contraction of the foot processes ultimately changing slit diaphragm architecture and allowing proteins to escape more easily into the urinary space [6]. Evidence that A II depolarizes podocytes by opening a chloride conductance related to cytoskeleton via an AT1 receptor is in line with such a possibility [7]. Increased glomerular permeability results in an abnormal protein trafficking through the glomerular capillary that contributes to progressive glomerular and tubulo-interstitial damage and eventually results in renal function loss and scarring [8].

Glomerular toxicity of ultrafiltered proteins

Recent data are in support of the possibility that the excessive protein load of the cells can be a factor underlying progressive podocyte injury [9]. Signs of enhanced uptake of plasma proteins by podocytes, as assessed by immunofluorescence analysis of IgG and complement C3, were found in remnant kidneys of rats with 5/6 renal mass reduction at 7 days after surgery, in a very early stage of disease. The granular intracellular pattern was entirely consistent with accumulation of proteins by endocytosis. By dual staining of sections of kidneys taken at 14 days after surgery, the abnormal expression of desmin, a marker of podocyte injury, was confined to the podocytes showing intracellular staining for plasma proteins. In addition, protein-laden podocytes showed loss of expression of synaptopodin, an actin associated molecule first detectable during foot process formation and thus an indicator of differentiated phenotype of the cell. These data were taken to suggest that the enhanced endocytosis of protein may concur to the perturbation of podocyte function that is currently recognized to play a major role in generating adhesive lesions and sclerosis. A causal link between protein load and podocyte dysfunction indeed was established by findings that the exposure of cultured podocytes to albumin (10 mg/ml) induced both loss of synaptopodin staining and expression and release of TGF-β1, a major stimulus for extracellular matrix production in the glomerulus. Moreover, the conditioned medium of IgG-loaden podocytes induced the expression of the myofibroblast-associated molecule alpha-smooth muscle actin in cultured mesangial cells. Such response was inhibited by the addition of neutralizing anti-TGF-β1 antibody [9].

Tubulo-interstitial toxicity of ultrafiltered proteins

Pioneering studies in rats with age-related proteinuria [10], or with adriamycin-induced nephrosis [11], found that protein reabsorption droplets accumulate in the proximal tubular cells. Evidence that protein accumulation was associated with focal breaks of tubular basement membranes, and extravasation of the tubular content in the renal interstitium led to suggest that plasma proteins may contribute to the tubulo-interstitial damage so frequently observed in animals or humans with long lasting, proteinuric nephropathies.

Both in vitro and in vivo, protein overload causes increased production of inflammatory mediators such as endothelin-1, monocyte chemoattractant protein-1 (MCP-1), RANTES (Regulated upon Activation Normal T-cell Expressed and Secreted), a chemotactic cytokine for monocytes and memory T-cells, and osteopontin [12]. The molecular mechanisms that lead to chemokine over-expression is mediated by NF-kB, a transcription factor that promotes nuclear translocation of the DNA [13,14]. There is in vitro evidence that albumin and IgG caused a dose-dependent increase in NF-kB activation in proximal tubular cells, an event that is followed by up-regulation of RANTES and MCP-1 [15,16]. In specimen of renal biopsies of patients with severe proteinuria, NF-kB activation has been shown in tubular cells, concomitant to up-regulation of pro-inflammatory chemokines [17].

Cytokines, growth factors and vasoactive substances may result in abnormal accumulation of extracellular matrix collagen, fibronectin and other components which are responsible for interstitial fibrosis. The pro-inflammatory mediators promote local recruitment of macrophages and lymphocytes, which in turn can stimulate the transformation of interstitial cells into myofibroblasts [18]. Proximal tubular epithelial cells can interact with interstitial fibroblasts to promote fibrogenesis via release of profibrogenic molecules [19].

Protein overload may also lead to an in situ activation of the complement system in proximal tubular cells associated with marked cytoskeleton alterations, increased production of superoxide anion and hydrogen peroxide, synthesis of proinflammatory cytokines and transmigration of T-cells across tubular epithelial cells [20]. Moreover, ultrafiltration of complement factors across the altered glomerular barrier may lead to complement C3 deposition and membrane attack complex formation (C5b-9) on the luminal side as well as C3 accumulation within proximal tubular cells [12]. These findings, combined to evidence that congenital absence of C6 limits interstitial inflammation and preserves renal function and structure in several proteinuric conditions [21], suggest a major role of the complement system in the pathogenesis of proteinuria-induced interstitial damage.

Direct toxicity of Angiotensin II

AII has an intrinsic toxicity that is independent of – and additional to – the nephrotoxic effects of increased protein traffic. AII modulates renal cell growth which in turn may contribute to tubulointerstitial injury [22]. Increased expression of c-fos and Egr-1, the immediate early genes whose activation precedes cell proliferation, has been shown in proximal tubular cells exposed to AII [23]. The peptide, acting through AII type 1 receptors, also induces hypertrophy in tubular cells by up-regulating the gene for transforming growth factor β1 (TGF-β1) which in turn leads to increased synthesis of collagen type IV [24]. Remodeling of the interstitial architecture may also occur as a result of transformation of tubular cells, an additional event promoted by the enhanced synthesis of TGF-ß1 stimulated by AII [25].

A II also stimulates the production of plasminogen activator inhibitor-1 (PAI-1), and may therefore further increase the accumulation of the extracelular matrix through inhibition of

its breakdown by matrix metalloproteinases, which require the conversion to an active form by plasmin [26]. By stimulating macrophage activation and phagocytosis, AII may enhance the inflammatory component associated with chronic renal injury [27]. AII up-regulates genes and stimulate secretion of peptides with chemotactic and vasoactive properties [28]. In experimental animals, repeated infusions of AII cause interstitial fibrosis and lead to the deposit of type IV collagen, a process that suggests the morphogenic effect of AII on tubulointerstitial structure [29]. Studies in a protein overload model of nephropathy [30] allowed to dissect the relative contribution of AII and proteinuria on chronic renal damage in animals with targeted gene deletion of the AII type 1A receptor (AT1-/-) as compared to wildetype mice (AT1 +/+). Normal animals not exposed to overload proteinuria acted as controls. AT1-/- animals developed proteinuria, renal failure and glomerular sclerosis although to a lesser degree than AT1 +/+ animals. In both models renal ET1 expression and synthesis was increased as compared to normal controls. These data confirm that both A II and plasma proteins have an intrinsic renal toxicity that is maximized when the two factors may play in combination [30].

Angiotensin II inhibition and nephroprotection

Animal studies

Evidence that A II blockade with an angiotensin converting enzyme inhibitor (ACEi) reduced proteinuria and slowed renal damage in a number of animal models of chronic renal disease [31,32], offered the opportunity, for the first time, to devise a treatment strategy which was not limited to passively accompain patients to their destiny of dialysis, but was aimed to preserve renal function as long as possible.

The antiproteinuric effect of AII inhibition has been initially attributed to the reduction of glomerular hypertension, but a direct effect on glomerular membrane permselectivity to macromolecules has been also demonstrated [32]. In in vitro and in vivo experiments ACEi prevent the expression of inflammatory mediators such as NF-kB, RANTES, MCP-1, and insulin-like growth factor [33]. This may result both from decreased exposure of glomerular and tubular cells to toxic effects of proteins and from direct inhibition of the proinflammatory properties of A II. In the remnant kidney model, the ACE inhibitor treatment limited the upregulation of TGF-β1 in podocytes, as well as the abnormal expression of alpha-smooth muscle actin in mesangial cells [33].

The development of a new class of RAS inhibitors, such as the Angiotensin Receptor Blockers (ARBs), has opened the perspective of novel strategies to achieve renoprotection. Indeed, several studies in different models of chronic renal disease uniformly found that ARBs may shear with ACEi a similar antiproteinuric and renoprotective effect [34].

Human studies

Over the last decade several clinical trials have evaluated whether the encouraging results achieved with RAS inhibitors in experimental animals translated in a real clinical benefit for humans with progressive nephropathies. In one of these trials, ACEi decreased the risk of doubling serum creatinine by 53% [35]. However, the large blood pressure difference between ACEi and placebo, made it impossible to separate the beneficial effects of better blood pressure control from any other effect specific to the inhibitor. Evidence for a specific renoprotective effect of ACEi was provided by the Ramipril Efficacy in Nephropathy (REIN) study [36-38]. In this study patients were randomly assigned to receive ramipril or conventional antihypertensive therapy to maintain diastolic blood pressure at 90 mm Hg or less. The trial was divided into two levels based on degree of baseline proteinuria (stratum 1; 1-3 gm/24 hour and stratum 2>3 gm/24 hours). The stratum 2 arm was stopped early because of greater efficacy of ramipril on preserving GFR. Despite a virtual identical blood pressure control in the two treatment groups, the ramipril group showed a slower rate of loss of GFR (mean monthly GFR decline 0.53 ml/min vs 0.88 ml/min) and a 50% lower incidence of ESRD as compared to controls [36]. Both effects were associated with a greater decrease in proteinuria (55% for ramipril vs no reduction for placebo). The rate of GFR decline was correlated negatively with the extent of proteinuria reduction [36] and positively with the level of residual proteinuria [39]. Of note, the renoprotective effect was seen across degrees of renal insufficiency, and patients in the lowest tertile of GFR (GFR 10 to 30 ml/min) also benefited from treatment with ACEi without a significant increase in the risk of hyperkalemia [40]. ACEi reduced the rate of decline of residual renal function even in patients with ESRD treated with peritoneal dialysis [41].

The African-American Study of Kidney disease (AASK), found a similar renoprotective effect also in patients – e.g. African-Americans with hypertensive renal disease – generally considered to poorly respond to ACEi therapy. Indeed, ramipril as compared to amlodipine decreased GFR decline by 36% and progression to clinical end-points by 38%, a finding that led the Ethics Committee to prematurely stop the amlodipine arm of the trial [42]. At final analyses, ACEi retained a superior renoprotective effect also as compared to beta blockade with metoprolol [43].

Prolonged ACEi therapy resulted in even more effective renoprotection. Nephrotic patients of the REIN study who, at completion of the core study, continued on ramipril for another two years (the REIN Follow-up study), enjoyed a progressive decrease in GFR decline up to a rate, approximately 1 ml/min/year, similar to that associated with normal aging [37]. After about 36 months no more patient progressed to the point of requiring dialysis [37]. Even more surprisingly, GFR slopes in sixteen of those patients progressively stabilized or were worsening so slowly that ESRD would be delayed beyond the patients' expected life time. Ten patients showed an improvement of GFR to the point that they might never reach ESRD [44]. On the contrary, patients originally on conventional treatment and switched to ramipril only on follow-up, despite a substantially reduced GFR decline, continued to progress and, in some cases, developed ESRD. Thus, ESRD risk reduction went from 50% in the core (18 months) to 300% in the follow-up (3-4 years) study, a finding consistent with a strongly time-dependent effect of ACEi [44].

Optimized angiotensin II inhibition to halt progression

Although encouraging, evidences from both experimental studies and clinical trials suggest that RAS inhibition postpones ESRD in most cases, but definitively prevent dialysis only in a minority of patients. Indeed, due to the current lag-time between starting treatment and achievement of remission, a substantial proportion of patients still progresses to ESRD before their renal function begins to stabilize. ACEi alone is sufficient to halt progression if therapy is started hearly, at GFRs still higher than 50 ml/min/1.73 m^2 [40]. To achieve this target at more advanced stages, a multimodal approach based on maximized RAS inhibition is needed. First, a low sodium diet may serve to activate the intrarenal RAS, which would maximize the response to ACEi or ATA. Diuretics may also achieve this, in particular when the response to RAS inhibition is blunted by sodium retention secondary to high sodium diet and/or severe renal insufficiency. However, maximized RAS inhibition mainly rests on the use of higher than antihypertensive doses of ACEi or ATA or of these two agents in combination.

High dose ACE inhibitor therapy

In Munich Wistar Fromter (MWF) rats with spontaneous disease, high dose ACEi given late during the animal's life when animals were already heavily proteinuric, decreased proteinuria and stopped the disease from progressing, as documented by a lower incidence of glomeruli affected by sclerotic lesions and less interstitial injury than untreated controls [45]. These data overall substantiated the results of previous morphological studies showing that ACEi, at doses exceeding the antihypertensive doses, imparted an additional benefit to glomerular structure, reversing the early glomerular lesions but not the advanced ones [46]. Sclerosis was also remodeled in aging rats by inhibiting the renin angiotensin system with an ATA given at high doses for six months [47]. The effect was attributed to the modulation of cortical cell turnover and inhibition of plasminogen activator-1 (PAI-1) expression.

In humans, lisinopril up-titrated to 40 mg/day (twice the standard antihypertensive dose for patients with normal renal function), despite no additional effects on blood pressure, further reduced proteinuria and, importantly, dose-dependently ameliorated the dyslipidemia associated with the nephrotic syndrome [48].

Combined ACEi and ARB therapy

Complementary or alternative to forced ACEi or ATA up-titration, is combined treatment with both agents [49,50]. The combination of an ACEi and ATA has been suggested as a way to maximize RAS blockade by affecting both the bioavailability of A II through ACEi and also by affecting its activity at the receptor level. ACEi have the additional properties of blocking the breakdown of bradykinin, a vasodilator that also stimulates nitric oxide production. ATA, do not affect the activity of the AT-R2, which appears to be important in vasodilation. Moreover they antagonize the activity of A II produced by non-ACEi sensitive enzymes such as chymase and other serine proteases [49,50]. The combination of these two drugs may be a way to block the effects of A II at the AT-R1 level, while achieving both increased bradykinin levels and activation of the AT-R2. This approach has recently offered a powerful tool to induce regression of renal disease at functional and structural levels. In a recently published study, the treatment with ACEi and ATA given to MWF rats during the interval between 25 and 40 weeks of age had remarkable effects [51]. Combined therapy completely reversed protein excretion and ameliorated renal plasma flow and the glomerular ultrafiltration coefficient. The reduction of the extent of existing structural damage was a key finding. Specifically the percentage of glomeruli with sclerotic lesions affecting less than 25% of the tuft decreased in respect to baseline, in the absence of increases in the percentage of glomeruli with more severe lesions. The degree of tubulointerstitial injury, including protein cast formation, macrophage infiltration and type III collagen accumulation, was also reduced by treatment. In this model the glomerular permselective dysfunction attributable to large, nonselective pores of the membrane precedes and may play role in structural injury independently of increased glomerular capillary hydraulic pressure. Given the effect of A II to disrupt the permselective function of the glomerular filtering barrier, the primary action of drug of ameliorating the functional barrier, presumably at the podocyte level, could contribute to prevent the detrimental effects of proteinuria and chemokine stimulation. Dual as compared to single drug RAS blockade provided superior benefit and partial regression of tubulointerstitial injury also in another model of severe, progressive renal disease, passive Heymann nephritis in uninephrectomized rats [52].

On the clinical ground, several studies found more proteinuria reduction with combined therapy than with ACEi or ATA alone [49,50]. This effect, however, was almost invariably associated with more blood pressure reduction with combined therapy, which did not allow concluding on whether the superior antiproteinuric effect of combined therapy depended on more RAS inhibition rather than on more blood pressure reduction. To dissect the relative contribution of these two mechanisms, we recently compared the antiproteinuric effect of combined therapy with halved doses of benazepril and valsartan with the effect of full doses of both agents used alone [53]. Finding that combined therapy reduced proteinuria more effectively than the two agents alone at virtually identical levels of blood pressure control provided consistent evidence of the intrinsic renoprotective effect of combined RAS inhibition. The benefit of combined therapy was more consistent, and clinically relevant, in patients with more severe, nephrotic-range, proteinuria. The superior, long-term renoprotective effect of combined vs single drug RAS inhibition was confirmed by the results of the COOPERATE study [54]. This study included 263 patients with non-diabetic, proteinuric nephropathies randomized to 3-year treatment with 3 mg/day of trandolapril, 100 mg/day of losartan or with halved doses of both drugs in combination. Eleven percent of patients on combination treatment reached the combined primary endpoint of doubling of serum creatinine concentration

Table 1. **Definitions of progression, remission and regression of proteinuric chronic nephropathies**

	Residual proteinuria (g/24 hours)	GFR decline (ml/min/1.73m2/year)	Renal structural changes
Progression	≥1.0	>1.0*	worsening
Remission	1.0-0.3	0.0-1.0*	stable
Regression	<0.3	<0.0	improving

* Physiological GFR decline associated with aging: 1.0 ml/min/1.73m^2/year

or ESRD compared with 23 percent of patients on trandolapril alone (hazard ratio 0.38, 95% CI 0.18-0.63, p = 0.018) and 23 percent of those on losartan alone (0.40, 0.17-0.69, p = 0.016). Thus, combined therapy reduced progression to the endpoint by about 60% as compared to single ACEi or ATA treatment. The most striking difference in groups was the much more consistent proteinuria reduction (versus pre-randomization values) on dual RAS blockade (76%) than on single ACEi (44%) or ATA (42%) treatment. Finding that the three treatment groups did not differ with respect to risk factors and showed the same reductions in blood pressure, combined to evidence that improved kidney survival was strongly associated with more proteinuria reduction, led further support to the hypothesis that proteinuria reduction may have an important pathogenetic role in the renoprotective effect of (dual) RAS blockade. Consistently with short-term data [53], the greater the proteinuria at baseline, the more was proteinuria reduction on follow-up [54]. Thus, in line with post-hoc analyses of the REIN study [36-40], patients with more severe disease at study entry and predicted to have a faster progression on follow-up, were those who finally benefited the most of renoprotective treatment. Hence, combined therapy was well tolerated, even in patients with advanced renal insufficiency, which provided further evidence that the practice of avoidance of ACEi, ATA or both to prevent further renal impairment and hyperkalemia in patients closer to ESRD is no longer justified [40]. Although good, however, these results show that a substantial proportion of patients with chronic nephropathies still continues to progress even on combined treatment.

Implementing angiotensin II inhibition with a multifactorial intervention: a way to achieve regression?

The RAS is the major, but not unique, determinant of progressive renal damage. Thus, targeting renoprotective therapy solely to the RAS may not be enough to achieve full remission/ /regression of chronic renal disease. Actually, experimental and human evidence is accumulating that both glomerulosclerosis and chronic tubulointerstitial injury, once developed, can be stabilized and even reverted when RAS inhibition is combined to treatments targeted also to non-RAS mechanisms. Thus – in analogy with other major medical conditions such as cancer or HIV infection – multi-factorial treatments may be required to definitely cure chronic nephropathies.

Experimental studies

In an animal model of nephrotic syndrome, the accelerated passive Heyman nephritis, a lipid-lowering drug added to ACEi and ATA further lessened the structural damage and ameliorated the outcome [55]. Combining ACEi, ATA and statin was therapeutic when given between 2 and 10 months. The triple drug therapy, despite similar blood pressure control as compared to less effective treatments, led to reduction of urinary protein to normal values and full prevention of renal failure.

Reduction of intrarenal leukocyte accumulation and expression of TGF-β may concur to mediate the beneficial effects [52]. Like TGF-β, other chemokines and growth factors of tubular and/or inflammatory cell origin such as interleukin-1 and tissue plasminogen activator (tPA), contribute to the production and regulation of glomerular and interstitial extracellular matrix. Some of these factors might play interrelated actions possibly relevant to regression of lesions. This appears to be the case, for instance, in mice lacking tPA that were protected against tPA-induced MMP9 gene expression and renal fibrosis [56]. Thus, besides RAS-blocking agents and statins, other drugs such as TGF-β inhibitors [57], vasopeptidase inhibitors [58], and agents to block immune and inflammatory reactions, such as mycophenolate mofetil [59], complement inhibitory molecules [60] and, in perspective, anti-TNF antibodies [61], are candidate components of the pharmacological cocktail aimed to achieve regression of the renal lesions.

Human studies

Evidence that regression of renal progressive disease and of the underlying lesion is achievable in humans can only be indirect, but it is fairly consistent and encouraging. Clinical findings of reduction of proteinuria to < 0.3 g/24h and increasing glomerular filtration rate indicate regression of proteinuric chronic nephropathy possibly reflecting improvement of renal structural changes [62] (*Tab. 1*). Combined therapy with ACEi, ATA, diuretics and statins blunted proteinuria and stabilized GFR for almost ten years in a young girl with nephrotic-range proteinuria who might otherwise have required dialysis within months [63]. In a series of ours, twenty-six patients whose proteinuria had been at least 3 g for more than 6 months, despite ACEi therapy, were given a standardized multidrug treatment including diuretics, ACEi, ATA, statins and non-dihidropyridine calcium channel blockers. Nineteen (73%) of these patients achieved full remission of proteinuria and their renal function stabilized over 24 months. Whether in parallel with clinical remission renal damage can also be reduced is still matter of investigation. In

support is the evidence by repeated biopsy for a trend of renal damage to regress leading to less mesangial expansion, more open capillaries and less interstitial fibrosis [64]. At least ten years were needed to reverse the lesions, which is entirely consistent with the concept that the timing of institution of therapy, beside the drug doses and combinations, is critical in the human setting exactly like in the experimental animal [65].

The implications of achieving regression

Regression of lesions may have significant impact on progressive renal disease and its sequelae. One might wonder why we should pursue the goal of improving renal structure and function, if we can already successfully stabilize the disease perhaps for a lifetime. First and most obviously, improving renal function may have a major effect in reducing the number of patients with chronic renal disease that progresses to ESRD. Any improvement of renal structure and function should also translate into less risk of ESRD for those who have less compromised renal function. This would apply both to young patients and to the elderly that may incur in more critical renal and cardiovascular risks. The economic impact of even a partial improvement would be enormous, as documented by findings that a 30% reduction in the rate of GFR decline would translate in more than 60 $ billions saved for providing renal replacement therapy to patients progressing to ESRD in the US by the year 2010 [66]. Finally, in certain settings, such as membranous nephropathy, focal and segmental sclerotic lesions predict worse prognosis. What would happen if we could revert them? Understanding the mechanisms by which a given lesion may regress and its relationship to function will be crucial to understanding the relevant renal cell biology and therapeutic targets. Once again, investigation in experimental models will prove indispensable to the new task and will clarify whether renal damage can be reverted to such a point that renal function may be fully prevented from worsen, and possibly improve.

Cardiovascular risk and cardioprotection in non-diabetic chronic renal disease

Cardiovascular risk in chronic renal disease

Even in the absence of classic risk factors such as hypertension, diabetes, dyslipidemia and smoking, patients with renal disease are at increased risk of cardiovascular events [67,68]. Cardiovascular morbidity and mortality linearly correlate with serum creatinine concentration and in patients with terminal renal failure may exceed that in the general population by ten to one hundred folds [68]. The HOPE study found that in patients with increased cardiovascular risk, even mild increases in serum creatinine (1.4 to 2.3 mg/dl) and microalbuminuria were equally strong-related risk predictors and were independent of each other. Patients with both risk factors had an incidence of cardiovascular events comparable to that of subjects with known coronary ischemic disease [69].

Several factors have been claimed to explain this strong renal-cardiovascular association. Renal insufficiency and proteinuria may play a direct pathogenetic role in the onset and progression of atherosclerotic disease, and may also amplify the effects of classic risk factors [70]. In particular, arterial hypertension (especially overnight), [71], dyslipidemia [72], hyper-homocysteinemia [73], and increased insulin resistance [74], may promote atherosclerosis and, in addition to cardiovascular remodeling [72], may increase the cardiovascular risk even in the very early stages of chronic renal disease. Moreover, microalbuminuria is considered to reflect a generalized endothelial dysfunction that may independently contribute to macrovascular disease [75]. Finally, there is evidence that RAS activation may be an independent risk factor for both renal and cardiovascular disease [76].

Angiotensin II inhibition and cardioprotection in chronic renal disease

Evidence that excess cardiovascular risk is associated with renal disease and that this excess may be, at least in part, associated with increased RAS activity [76] provides a further, strong rationale to RAS inhibition therapy in patients with chronic nephropathies. Although ad hoc studies are missing, post-hoc analyses of the Heart Outcomes Prevention Evaluation (HOPE) trial [69], that included almost one thousand patients with mild-moderate renal insufficiency, allowed to evaluate the impact of renal function on the cardioprotective effects of ACEi therapy. Actually, ramipril uniformly decreased the overall cardiovascular risk across quartiles of basal serum creatinine, and reduced overall mortality, cardiovascular mortality and hospitalization for heart failure even more effectively in patients with serum creatinine $\geq$1.4 mg/dl than in the overall study population. On the same line, in the renal and cardiovascular treatment program introduced in late 1995 into an high-risk Australian Aboriginal community, ACEi therapy resulted in a 50% reduction in rate of natural (mostly cardiovascular) deaths and 57% reduction in ESRD events [77]. Large part of renal and cardiovascular events occurred in subjects with evidence of renal disease (macroalbuminuria) and the beneficial effect of ACEi inhibitor therapy was largely driven by the remarkable risk reduction achieved in this subset of patients [77]. Despite these encouraging results, many physicians still have safety concerns to use RAS inhibitors in patients with renal insufficiency. However, both the REIN [36-38] and the HOPE [78] study failed to detect any association between renal insufficiency and premature ramipril withdrawal because of adverse events. In particular, in the HOPE study the incidence of ramipril-related symptomatic hypotension, cough, and angioedema was independent of basal serum creatinine levels. Moreover, at comparable levels of serum creatinine, the incidence of premature withdrawal because of hyperkalemia or worsening renal function was comparable on ramipril and on placebo. Thus, in patients with renal insufficiency ACEi therapy is even more cardioprotective than in the general population and is well tolerated. Thus, it should not be withheld simply because of a moderate (<30%) elevation in serum creatinine concentration. Higher increases should arise the suspicion of a concomitant ischemic kidney disease or of an overzelous diuretic therapy resulting in decreased effective arterial volume and RAS activation. Response to treatment is less predictable in renal patients with congestive heart failure. In most cases, the improvement in systemic hemodynamics achieved by RAS inhibitor therapy may also result in an improvement in kidney function. However,

when kidney perfusion and ultrafiltration are largely dependent on an activated (intrarenal) RAS system – such as in patients with severe cardiac dysfunction and remarkably decreased effective arterial volume – RAS inhibition may result in kidney hypoperfusion and dysfunction. In most cases, however, this is a transient effect and empirical down-titration of the ACEi or ATA dose and/or of concomitant diuretic therapy may help achieving a balance between the systemic and renal effects of ACE inhibition that may result in improved hemodynamics and kidney function [79]. Of note, the long-term benefit of RAS inhibition therapy appears similar in patients with and without substantial elevations in serum creatinine levels [79].

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Principles of family practitioners and nephrologists collaboration

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Abstract

There is a need to establish clear standards of cooperation family doctors with specialists and arrange specialist service for family practitioners to improve a care for patients with urinary system diseases.

Our paper presents attempt on the estimation of medical care system reform and state of general practitioners with specialists cooperation level as well as draw conclusions regarding possibilities of specialist and primary care contacts improvement and by this – increasing of comprehensive patients care quality.

Family doctor never tried to replace with a specialist. Only constructive and professional cooperation specialists with family doctors can secure proper care for patients.

Key words: general practitioner, nephrologist, collaboration.

Health Care System (HCS) in Poland is in continuous process of transformation. Before 1989 we had a system based on hospital, near-hospital specialist centers and local specialist units. There were no Family Medicine/General Practice specialists at all – the role of GPs was replaced by a team of internal medicine specialist and a paediatrician (sometimes with laryngologist, gynaecologist, dentist etc.). Patient was assigned to the nearest health care unit and couldn't choose a doctor. Almost all health care units were a public property [1-4].

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The growing number of non-public primary care practices especially family doctor practices originated from the transformation of the National Health Care system in Poland which started in 1998, giving independence to health care practices, and forming and strengthening the structure of the health care organizational body. This meant structures health care system could negotiate contracts with public and non-public practices. Further stages of health care system reforms aimed to strengthen the role of family medicine and put the focus on the promotion of family doctors who were able to bid competitive offers for providing health services [4].

The aim of reforming the Polish National Health Service is to improve the general health of the population and run effectively polish care system. The target number of GP in Poland is 20000 for 40 mln population.

Pharmacies, dental practices and a quite a lot of primary and secondary care units are private. Most of the hospitals are still public (local government's) property [1-4].

Primary care in Poland is to be provided by family medicine specialist. The insured patient chooses a doctor (signs a declaration). Family doctors are contracted on capitatation fee and they are responsible for population reported on their list (max 2500 patients), differently from specialists who have fee for service system. Family doctor signs one year contract with Regional Department of National Health Fund (public health insurance unit) and gets capitation fee monthly.

For now we have no certification/recertification system in family medicine [4].

Family Practice is open five days a week (from Monday to Friday) with working hours between 8 a.m. and 6 p.m. Out of hours patients usually report to Emergency Care Unit.

The out of hours services vary in different parts of our country. There are 3 general models:

a) services provided by emergency units,
b) services provided by family doctors/specialists on additional contracts with other family doctors in special "out of hour" centers,

c) services provided by GPs themselves at their own practice.

There is a document, written by College of Family Physicians in Poland which describes all duties of family practitioners – it leaves a wide range of competences in the hands of family practitioners. A reference document for constructing of undergraduate and postgraduate training program in family medicine has been written. But, in fact, the real duties of GP depend on a signed contract [1-4].

There is the referral system to specialist's care in Poland. It is required for the patient to be referred to the specialist excluding the following: ophthalmologist, dermatologist gynaecologist (obstetrician), oncologist and psychiatrist. Cooperation between GP and specialist is rather difficult because of low availability of specialist care, long waiting list for consultation, lack of a good system of information exchange concerning referred patients.

There are three levels of secondary care – provided by local specialist care, provided by specialist centers (mainly situated in large specialist hospitals) and university/medical school centers [4].

Specialists also sign contracts with public Fund – they have "fee for service" payment system (but there is a maximal number of medical procedures during a contracted period) [4-6].

Patient who has obtained a referral from GP can choose any secondary care unit to take care of her/him. The most expensive procedures (e.g. cardiosurgery, chemotherapy) are paid directly from central budget of Ministry of Health.

Choosing one doctor as your primary health care professional is your best insurance for better health care. By letting this doctor coordinate health care needs, the patient can be assured of the highest quality care. This means the doctor will know the complete health history, what kinds of medications the patient is on, how a new medication might interact with existing medications, and, if necessary, what kinds of specialists he may need. With this knowledge, the doctor will be better equipped to make the best recommendations when the patient need care especially – specialist consultation or diagnostic [2].

There is still a need to establish clear standards of cooperation family practitioners with nephrologists during diagnostic and treatment of kidneys' diseases [2,7].

The family practitioner should be able to conduct diagnostic and treatment:

- acute and uncomplicated urinary system's infections
- nephrolithiasis
- acute glomerulonephritis
- polycystic degeneration of kidneys
- uncomplicated anomalies of kidneys
- chronic renal failure (first stage, second – after the nephrologist's consultation)
- primary hypertension
- secondary hypertension (after the specialist consultation)
- gouty diathesis
- asymptomatic bacteriaemia
- complications of glomerulonephritis (between the nephrologist's consultations)
- the stage between haemodialyses (acute conditions)
- the stage during peritoneal dialyses (acute conditions).

The family practitioner has following diagnostic tests at disposal to fulfill the competences during collaboration with the nephrologist:

- urine analysis
- urea and creatinine concentration
- creatinine clearance
- electrolytes: Na, K, Ca, PO_4, Mg
- Fe concentration
- ESR, morphology
- ASO, complement-binding test
- urine culture with antibiogram
- abdominal scout film
- USG of abdomen
- concentration of cholesterol, HDL, LDL, triglyceride
- proteinogram
- gasometry
- concentration of GOT, GPT, GGTP
- concentration of bilirubin
- concentration of glucose
- markers of HBV and HCV infections.

The main tasks of the family practitioners are:

- knowledge of symptoms and diagnostic tests in kidneys' diseases
- follow-up of specialist recommendations
- knowledge of nephrotoxic medications
- doses modification due to creatinine clearance
- health promotion and prophylaxis in kidneys' diseases.

The proper functioning family practitioner should:

- start early diagnostic and therapy of urinary system diseases
- continue the therapy started by nephrologist
- know when and with which diseases to refer the patient to a nephrologist
- know which diagnostic tests are indispensable before a nephrologist consultation
- know principles of water balance and creatinine clearance estimation
- know principles of nutrition of patients with urinary system diseases
- know causes of urinary system diseases
- avoid anomalies of kidneys
- propagate the health promotion and prophylaxis of urinary system diseases
- collaborate with nephrologists during comprehensive care for patients with urinary system diseases.

There are very few relationships as important as those which bind a physician with a patient. To find a qualified doctor worthy of the patient confidence, requires time and effort. It is worth while, since this will give years of quality life, or even sometimes save the life. In addition to a great integrity, several factors and qualities must be joined together so that you will be completely satisfied with the choice [1].

A good doctor (a family practitioner as well as a specialist) is personally concerned with regularly advices on preventive medicine, including an anamnesis (personal history), i.e. personal medical history, a physical examination, and done with respect, confidence in a doctor who prescribes paraclinical examinations (laboratory tests, radiology), without having initially made anamnesis and physical examination, physical examination whose extent depends on anamnesis result [1].

A family practitioners and a nephrologists are partners in the care of patients: their duty to the patient comes first. They works for the good of the patient, not that of the government, an insurance company, or a managed health care bureaucrat.

Generally, any physician (a family practitioner and a specialist), officially agreed or not, knows extremely well that he is not a recipient in the contract which binds the policy-holder and the insurer and he is not co-signatory. In fact the physician does have and can have only one master: his patient especially during the care for the patients with so complex problems that urinary system diseases are.

The aim of reforming the Polish national health service is to improve the general health of the public and the effective running of the health service, as experience from western European countries and Poland shows, it is advantageous for the development of family medicine [4-6].

There is still a need to establish clear standards of cooperation family practitioners with specialists and arrange specialist phone service for family practitioners.

Family doctor never tried to replace with a specialist. Only constructive and professional cooperation specialists with family doctors can secure proper care for patients especially with the urinary system diseases.

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Application of LDL-apheresis and immunoadsorption in kidney diseases

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Abstract

Plasmapheresis is one of the methods of extracorporeal blood purification used for many decades for the treatment of different kidney and extrarenal diseases, mainly of autoimmunological nature. The main disadvantage of this method is the lack of selectivity and the risk of infections associated with plasma used for supplementation. Hence, the efforts are made to establish an alternative blood purification treatment that might be used in renal diseases instead of plasmapheresis. These alternative methods should be more selective in certain pathogenic factors elimination and result in less risk for patient, both acute and delayed. Recently two such methods were applied more frequently to everyday nephrological practice, i.e. LDL-apheresis and immunoadsorption. The present paper aims to review the current state of knowledge regarding use of two mentioned methods in kidney diseases. Despite their very high costs both of them if used early in certain, refractory nephropathies may ameliorate their clinical course and significantly improve the prognosis. In addition they may significantly reduce the overall costs of therapy due to avoidance of unnecessary immunosupression, prolonged hospitalization and finally – costs of postponed renal replacement therapy.

Key words: plasmapheresis, LDL-apheresis, immunoadsorption, kidney diseases, renal transplantation.

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Introduction

Plasma exchange (PE; plasmapheresis) belongs to the group of methods commonly called extracorporeal blood purification techniques. They are used not only for the treatment of kidney diseases, but also in many other disorders, mainly from the area of neurology and toxicology. In some diseases this method was proven to be very effective (anti-GBM glomerulonephritis, Guillan-Barre syndrome, hemolytic-uremic syndrome, recurrence of certain glomerulopathies in transplanted kidney), whereas in others its effectiveness remains doubtful and lacks good support with prospective clinical trials. Although relatively safe, traditional plasma exchange carries certain risk for the patient, both acute (related to the procedure itself), as well as delayed (risk for transmission of certain viral infections with the fresh frozen plasma used as the supplement fluid appears the most important one). Unfortunately, during therapeutic elimination of immunoglobulins or immune complexes that might be involved in the pathogenesis of the disease, this method exposes the risk related to non-selective, uncontrolled elimination of other circulating agents, including blood coagulation factors, hormones etc. Thus the researchers focused on the development of new techniques that might provide more selective techniques targeting certain circulating pathogenic agents without the concomitant removal of physiological factors.

Among methods used in extracorporeal blood purification two of them attract a special attention from nephrologist's point of view as potential tools in the treatment of renal diseases. These methods are LDL-apheresis and immunoadsorption.

LDL-apheresis

Selectivity of LDL-apheresis

Different methods of apheresis possess different selectivity for pathogenic factor removal. The simplest one is of course plasmapheresis with no selectivity at all. Double-filtration plasmapheresis (DFPP) is also characterized by relatively low

selectivity, as plasma proteins are eliminated depending on their molecular weight only. Another method frequently used, heparin-induced extracorporeal lipoprotein precipitation (HELP) allows eliminating not only LDL-cholesterol, but also other factors that precipitate in low pH in the presence of heparin. As these factors include lipoprotein (a) and fibrinogen, this cannot be considered as a side effect, but rather an additional benefit of the procedure. Indeed, HELP is very efficient in elimination of both mentioned proatherogenic agents. The efficacy of fibrinogen elimination (highest among all extracorporeal LDL-cholesterol removal techniques) limits the volume of plasma that may be processed during single procedure to 3000 ml per session to avoid pronounced fibrinogen depletion.

The elimination of different plasma proteins deserves special attention in case of dextran sulphate, the basic ligand used for LDL-apheresis. In his interesting review Kojima divided these substances depending of the mode of interaction with the column into four groups: proteins containing apolipoprotein B (LDL-cholesterol, lipoprotein a), proteins that become activated in the initial contact phase of the intrinsic coagulation pathway (prekallikrein), lipophilic proteins that adhere to the phospholipid portions of LDL-cholesterol already bound to the column (coagulation factors VIII, V, VII, X, vitamin E) and other adhesive proteins (von Willebrand factor, fibronectin) [1]. Again, the elimination of most of mentioned proteins is rather positive, than undesirable effect, as most of them are proatherogenic. As it has been shown in comparative studies, all LDL-apheresis techniques to the different extent eliminate also HDL-cholesterol and triglicerydes, although these effects are transient [2].

LDL-apheresis in refractory hypercholesterolemia

Although the aim of this paper is to review the role of LDL-apheresis in renal disease, we should also refer briefly to its use in other diseases. Originally, LDL-apheresis was established to treat familiar homo- or heterozygous hypercholesterolemia refractory to conventional treatment (i.e. diet plus lipid-lowering drugs, mostly HMG-CoA inhibitors). The data available on the influence of LDL-apheresis on lipid profile and on the possible clinical symptoms, although convincing, are usually obtained in small groups of patients. It is obvious, as apheresis may be performed only in highly selected populations, in which both diet and lipid-lowering drugs appeared to be ineffective [2-5]. Another important consideration is that these studies cannot be blinded for obvious reasons [2]. Nevertheless most of the trials provide the data that confirm the efficacy of LDL-apheresis, especially when combined with pharmacological treatment. This efficacy is obtained not only in terms of serum lipid concentration lowering, but also in the prevention of atherosclerosis progression, decrease in the onset of new cardiovascular and cerebro-vascular events, as well as substantial reduction of mortality [2-5]. Some studies suggest that even slight regression of atherosclerosis may be achieved under the treatment with LDL-apheresis [3,5].

LDL-apheresis in kidney diseases

Initial concept for LDL-apheresis was to use it as a lipid-lowering treatment (lipoprotein elimination) in refractory (mostly familial) hypercholesterolemia. However, this method was also proven to be effective in the treatment of nephrotic syndrome (NS). The use of this method in NS is logical, as this group of diseases manifests with severe dyslipidaemia. Interestingly, LDL-apheresis was effective not only in symptomatic lowering of plasma lipids, but also influenced the course of nephropathies of different origin. In this review we aimed to focus mostly on the impact of LDL-apheresis on the clinical course of various primary and secondary nephropathies.

There are only few indications in renal patients, in which the usefulness of LDL-apheresis seems to be proven, although mostly in non-randomized observations performed in small-sample size populations. These indications include: primary focal segmental glomerulosclerosis (FSG), recurrence of FSG in transplanted kidney and lupus nephritis. Anecdotal reports indicate that many other glomerular diseases irrespectively from the type might be successfully treated with LDL-apheresis, when other therapies were ineffective.

Yokoyama and co-workers showed a significant decrease of daily protein loss and increase in GFR after the course of LDL-apheresis among 14 patients with FSG refractory to previous steroid treatment. Six procedures performed over 3 weeks were sufficient to achieve the improvement in GFR, the reduction of daily protein loss and normalization of lipid profile for more than 6 months of follow-up. In addition, repeated biopsies performed 3 months after LDL-apheresis therapy revealed the regression of morphological lesions when compared to initial (pre-treatment) samples. As one could expect the response to treatment was better in patients with less severe sclerosis on initial biopsy, but with more severe nephritic syndrome at the treatment initiation [6]. In the study of Musso et al. an amelioration of symptoms of nephrotic syndrome due to FSG or minimal-change nephritis resistant to previous treatment was accompanied by decreased intensity of mesangial staining for ApoB and reduced number of macrophages infiltrating the glomeruli in patients responding to LDL-apheresis therapy [7]. Many case reports confirm, that LDL-apheresis may be successfully employed in the treatment of FSG and that along with lowering of serum lipid profile proteinuria decreases, serum total protein and albumin levels increase and renal function improves [8,9]. The concomitant use of HMG-CoA inhibitors may additionally enhance the effect of LDL-apheresis [9,10]. The use of mentioned drugs is also advisable to avoid a rebound effect (i.e. the return of serum lipids to pre-treatment level after certain period of time). In addition, this group of drugs could probably improve the clinical course of certain nephropathies in lipid lowering-independent mechanisms, probably associated to its anti-inflammatory and antithrombotic properties.

The employment of LDL-apheresis in lupus-associated nephropathy should probably be limited only to patients who are resistant to other therapies and those with rapid clinical and morphological progression to renal failure. Daimon and co-workers described such a patient with rapidly progressing glomerulonephritis in the course of systemic lupus, with presence of crescents in 11 out of 22 glomeruli in renal biopsy specimen, who needed dialysis due to rapid deterioration of renal function. The initial use of plasmapheresis was sufficient to recover renal function and quit renal replacement therapy, although did not protect the patient from severe nephrotic syndrome. This

state was further treated with LDL-apheresis using Liposorba columns and later on – with DNA-adsorption using Selesorb columns [11]. This case report provides an excellent example for an 'integrated' extracorporeal treatment using four (including hemodialysis) blood purification techniques for treatment of severe systemic lupus.

LDL-apheresis may also be effective in the treatment of nephrotic syndrome associated with the recurrence of glomerulonephritis in transplanted kidney. This method appears to be especially effective in recurrent FSG [12].

Effectiveness of LDL-apheresis was also proven in other diseases associated with nephrotic syndrome resistant to immunosuppressive therapy, such as minimal change nephritis although vast majority of reports are limited to small groups of patients or even single cases [13-15]. Interestingly, LDL-apheresis may also ameliorate renal function in diabetic nephropathy [16,17].

LDL-apheresis: mechanisms of action in glomerular diseases

Several mechanisms in which the lowering of serum lipids may improve kidney function and slow down or even reverse the progression of renal insufficiency were proposed. LDL-cholesterol in its oxidized form may induce the formation of foam cells, thus accelerating inflammatory process and glomerular damage [6,7,15,18]. Another possible mechanism is binding of lipoproteins to polyanionic glycosaminoglycans in the mesangial matrix and glomerular basement membrane with further modification of their structure, electrical charge, and in consequence – permeability [19]. Oxidized LDL particles and lipoprotein (a) stimulate renin release by the juxtaglomerular cells, which may additionally contribute to the progression of renal failure in both hemodynamic and inflammatory mechanism (angiotensin II is one of the potent stimuli of TGFβ release, which in turn is the most important factor that accelerates renal interstitial fibrosis) [19,20]. On the other hand, reduction in proteinuria, despite the mechanism, diminishes LDL-cholesterol synthesis in nephrotic syndrome [10].

Although relatively selective, LDL-apheresis removes also coagulation factors V, VIII and von Willebrand factor. In addition, after LDL-apheresis the significant reduction in urinary excretion of thromboxane A_2 and B_2 was noticed. These changes suggest that improvement of renal function after the course of treatment with LDL-apheresis may at least in part depend upon the improved blood rheology in renal microcirculation [14,15].

Interesting observation was made by Muso et al., who found, that the treatment with LDL-apheresis not only increases creatinine clearance in patients with initially impaired renal function, but also leads to substantial decrease of abnormally high glomerular filtration rate to the normal range in nephrotic subjects [15]. This observation is very important, as the glomerular hyperfunction (hyperfiltration) is one of the important factors responsible for glomerular damage.

It has been proposed, that lipids removal may increase the response to concomitantly used immunosuppressive therapy [6, 14]. As it was suggested, after LDL-apheresis an up-regulation of receptors for steroids may occur [14,15]. Cyclosporin-LDL complexes are also bound to LDL receptors, hence the removal of lipids with LDL-apheresis may increase the availability of this drug [22].

Another mechanism that leads to amelioration of kidney function after LDL-apheresis in different nephropathies may be the improvement of endothelium-dependent vasodilatation after this procedure noticed in hypercholesterolemic patients [23]. Vasodilatatory properties of blood vessels are seriously compromised in patients with renal diseases; the restoration of this function with LDL-apheresis may possibly contribute to the improvement of renal function due to improvement of local blood circulation.

The real efficacy of LDL-apheresis is largely confounded by concomitant treatment with immunosuppressive drugs. From this point of view an interesting observation was maid by Yokoyama et al., who performed LDL-apheresis in patient with FSG previously naive to immunosuppression [24]. Patient described in the cited case report experienced marked improvement in renal function and significant decrease in urinary protein loss from initial 5.31 to 0.87 g/day after initial course of 6 LDL-apheresis procedures without concomitant treatment with immunosuppressive drugs. This clinical improvement was accompanied by regression of some histological lesions in repeated kidney biopsy. Patient was maintained on LDL-apheresis treatment alone performed twice a month [24]. Of course this data, although very interesting, cannot suggest the use of LDL-apheresis alone as a routine treatment. Nevertheless, this observation suggests that the discussed method may be useful also without concomitant immunosupression.

Immunoadsorption

Another technique that recently deserved special attention of nephrologists is immunoadsorption (IA). This technique allows eliminating immunoglobulins and other circulating agents that might be involved in the pathogenesis of different diseases, mostly of immunological origin, in the relatively selective manner. Selectivity is largely dependent on the type of the membrane and especially – the type of ligand used for adsorption. These adsorbents include staphylococcal protein A (SPA), tryptophan, phenylalanine, monoclonal sheep anti-IgG-human immunoglobulin, dextrane sulphate [25-29]. The types of membranes and ligands used for LDL and IA as well as selected indications for treatment in renal and extrarenal diseases are summarized in *Tab. 1*. Depending on the type of the ligand used and the type of circulating pathogenetic factor the elimination this factor may be based on immunological, chemical or electrostatic interaction. Most of the IA techniques utilizing mentioned ligands are designed to eliminate circulating antibodies and immune complexes. Indeed, these methods may really be selective in elimination of certain plasma components. As it has been shown for dextran sulphate in *in vivo* experiment, this ligand was able to adsorb 62% of passed anti-DNA antibody, 47% of cardiolipin and 26% of immune complexes, whereas no adsorption of total protein, albumin, immunoglobulin G nor C3 complement component was noticed [30]. Of course this theoretical selectivity is not fully

sustained *in vivo* – the substantial amounts of IgG or albumin are also lost during the procedure [27,31]. Interestingly, as it was shown in patients with lupus nephritis, IA possesses also immunomodulatory properties, as it is able to restore the initially decreased complement level [32,33]. IA selectivity may be further improved using more specific ligands, such as anti-β_2-microglobulin antibody or hexadecyl alkyl chains containing columns (designed exclusively to eliminate β_2-microglobulin in dialysis patients), or acetylocholine receptor peptide columns in the treatment of myasthenia [28,34,35]. Immunoadsorption using specific LDL-apolipoprotein B binding antibodies or dextran sulphate are also used for the LDL-chlesterol elimination, as it was discussed in the previous section of current review.

Immunoadsorption in primary and secondary nephropathies

Similarly to the statement made when discussing LDL--apheresis, also the use of IA is especially important in fast – progressing, treatment – resistant nephropathies with poor renal and overall prognosis. This is especially true for those nephropathies which manifest clinically as rapidly progressive glomerulonephritis and morphologically as crescentic nephropathy. In one of the early reports, Palmer et al. described significant improvement of renal function in 10 patients with crescentic glomerulopathy already on dialysis. In all cases immunoadsorption coupled with immunosupression resulted in recovery of renal function and dialysis – independency. Simultaneous resolution of glomerular crescents was observed [36]. Similar results were also presented in the number of case reports, describing both patients with primary crescentic nephropathies and Goodpasture syndrome [26,37]. As one could expect the presence of 'early', cellular, but nor fibrous crescents is probably the *sine qua non* condition for therapeutic success in IA, as it happens for 'classical' immunosupression.

Large group of diseases that may manifest as crescentic (rapide progressive) glomerulonephritis are ANCA-positive vasculitides. Matic et al. reported 3 cases of Wegener's granulomatosis with severe renal and other systemic involvement successfully treated with IA. In all patients the clinical improvement was accompanied by complete elimination of circulating c-ANCA antibodies [25]. However, in the prospective randomized trial comparing IA versus plasmapheresis in the treatment of rapidly progressive glomerulonephritis (RPG) in patients with at least 50% of glomeruli with crescents Stegmayr and co-workers failed to demonstrate any difference in patients' outcome between the two treatment groups [38].

The studies performed to date clearly indicate that 'classical' plasmapheresis is not effective in the treatment of lupus erythematosus. Several studies showed its relative usefulness as a symptomatic treatment relieving acute symptoms of flares, but none of them was able to demonstrate the independent impact of PE on survival. In opposite, IA appeared to be useful in the treatment of severe cases of systemic lupus, probably because it allows treating much larger volumes of plasma. Although the large prospective studies are still lacking, the preliminary clinical reports are very promising. Remission was achieved in 7 out of 8 patients with drug – resistant SLE treated with immunoadsorption onto protein A, reported by Braun and co-workers. In all patients the substantial amelioration of symptoms from different body systems was achieved, including arthritis and polyserositis, decline of proteinuria and serum creatinine concentration. Clinical improvement was accompanied by significant decrease of circulating plasma autoantibodies. Interestingly, IA led to restoration of initially decreased complement levels [32]. The same authors described the case of critically ill patient with lupus (renal WHO class IVd), non-responding to any drug treatment applied. She was successfully treated with IA after previous discontinuation of all immunosuppressive agents when became dialysis dependent and presented with grand mal seizures. After two weeks of repeated IA dialysis treatment was stopped; renal biopsy was performed 9 months later and only minor morphological abnormalities were found [33]. Another report showed significant improvement in overall SLE disease activity index and in most of the clinical manifestations such as skin lesions, leukopenia and proteinuria in 19 SLE patients treated with mean number of 3.7 IA procedures using dextran sulphate as a ligand [39]. The use of IA together with steroids and/or cyclophosphamide allowed for significant reduction of total dose of immunosuppressive agents and appeared to be an additive synergistic therapy [39].

Effectiveness of IA in elimination of anticardiolipin antibodies appears to be a major advantage in the treatment of SLE (and possibly – in primary anti-phospholipid syndrome). The onset of anti-phospholipid syndrome leads to very serious complications of the disease, including marked mortality among patients, mainly due to thrombosis, and habitual abortions in pregnant women. Hence, the new alternative for treatment of this dangerous disease may be of great importance in terms of possible reduction of mortality and improved outcome for pregnancies [33,40,41].

The interesting results of the treatment with IA in nephrotic syndrome of primary and secondary origin were presented in 1999 by Esnault et al. They used repeated IA to protein A columns in 9 patients with membranous glomerulonephritis, IgA glomerulonephritis, amyloidosis and diabetic nephopathy. Significant reduction of proteinuria from the mean 12.6 ± 5.49 to 3.35 ± 2.2 g/24 h (75.4% decrease) was achieved. It is worth to emphasize that authors obtained significant amelioration of proteinuria also in the disease of non-immunological pathogenesis, namely diabetic nephropathy [31].

Immunoadsorption in solid organ transplantation

Except of the usefulness of IA in the treatment of 'native' kidney diseases this technique seems to be promising in renal transplantation. First, the discussed method may help preparing high-risk patients for transplantation. Haas and colleagues used IA to lower the level of panel reactive antibodies (PRA) in patients awaiting retransplantation. They performed up to 24 sessions in pre- and early posttransplant period in 20 highly sensitized patients (median PRA 87%), achieving excellent patient and graft survival [42]. Second, severe complications of transplantation may also be managed using IA. Boehmig et al. treated acute humoral renal transplant rejection in ten patients displaying C4d deposits in peritubular capillaries on renal biopsy, using immunoadsorption onto protein A (median number of sessions equaled 9), together with standard anti-

Table 1. **Selected ligands used for immunoadsorption and apheresis, their trade names and indications for clinical use**

Procedure	Ligand	Trade name/Manufacturer	Therapeutic Indication/Factor eliminated
LDL-apheresis (HELP)	Physical factors (low temperature), heparin	B. Braun	Elimination of LDL-cholesterol, lipoprotein (a) and fibrinogen
LDL-apheresis	Anti-Apo B antibodies	DALI system, Fresenius	Lipoprotein elimination
	Dextran sulphate (LA-15, LA-40)	Liposorba, Kaneka	Elimination of lipoproteins, LDL-cholesterol, apolipoprotein B, lipoprotein (a)
Immunoadsorption	Dextran sulphate	Selesorb, Kaneka	Non-specific binding and elimination of antibodies and immune complexes; used in myastenia, Guillain-Barre syndrome, prophylaxis of acute rejection in AB0 blood groups incompatibility and in the presence of anti-HLA antibodies, immunovasculitis, Goodpasture disease and syndrome, SLE, habitual abortion, antiphospholipid syndrome, infiltrative ophtalmopathy in Graves-Basedow syndrome, primary and recurrent FSG (permeability factor elimination)
	Staphylococcal protein A (SPA)	Immunosorba, Fresenius Prosorba, Kaneka	
	Anti-IgG antibodies	Therasorb, PlasmaSelect; Baxter	
	Fenylalanin	PH-350	
	Tryptophan	TR-350	myastenia, autoimmune polineuropathy, rheumatoid arthritis (rheumatoid factor)
	Polimyxin B		Sepsis (elimination of endotoxines, cytokines, chemokines)
	Albumin	MATISSE, Fresenius	
	Hexadecyl alkil polymer	Lixelle, Kaneka	β_2M-amyloidosis
	Monoclonal anti-β_2M antibody; recombinant fragment scFv of anti-β_2M antibody		
	Fibrinogen-binding pentapeptide	Rheosorb, PlasmaSelect	Fibrinogen, fibrin, fibrinogen degradation products; impaired blood rheology, hypercoagulability
	Anti-acetylocholine receptor antibodies	Medisorba MG, Kuraray Medical Inc.	Myasthenia gravis
	Resins: Amberochrom Amberlit		Sepsis/removal of: 80% TNFα 100 IL-1α, IL-1β, IL-8 40% TNFα

rejection therapy. In eight out of 10 treated patients prolonged normalization of graft function for a mean period of 14.2 +/- 7.1 months has been achieved [43].

The recurrence of certain nephropathies (or their de novo onset) appears to be an emerging problem in renal transplant recipients. Although not routinely used in primary FSG, immunoadsorption seems to be promising in the treatment of recurrent FSG after renal transplantation. The recurrence of mentioned nephropathy is related to not completely recognized circulating 'permeability factor' that impacts on permeability properties of glomerular filtrating membrane. Bussemaker et al. described the patient with severe nephrotic syndrome (urinary protein loss of up to 35 g/d, with advanced glomerular lesions on graft biopsy seven months after transplantation), in which IA to tryptophane (11 consecutive sessions) was able to reduce proteinuria to 2.0 g/d and to restore serum albumin level and normal renal function [27]. Other authors confirmed the successful outcome of recurrent FSG in renal transplant recipients treated with IA [44,45]. Belson et al. described a 9-years old patient who started plasmapheresis since 12th posttransplant day because of severe nephrotic syndrome due to recurrent FSG, followed by immunoadsorption on protein A column since 8th post-transplant month and remained on this treatment for up to 60 months [46].

Initial results with this procedure are so encouraging, that in the EDTA guidelines for renal transplantation released in the year 2000 immunoadsorption on protein A or anti-IgG column as well as plasmapheresis were recommended as measures for recurrent FSG in renal transplant recipients (Guideline IV.2.5.A) [47].

"Non-renal" indications for immunoadsorption

Another promising indication of immunoadsorption may be the removal of β_2-microglobulin. This agent is responsible for many serious complications in patients on long-term dialysis. 'Traditional' hemodialysis treatment with bioincompatible membranes is not sufficient enough to eliminate this low molecular weight protein. The experimental use of the column containing anti-human β_2-microglobulin antibodies in vitro was sufficient to eliminate this agent to the level below the detectable limit of assay [34]. Another method for selective elimination of β_2-microglobulin, based on hexadecyl alkyl chains was also effective in adsorption of endotoxins in an in vitro model [35].

Protein A-based IA was also effective in the removal of anti-platelet antibodies in idiopatic thrombocytopenic purpura, anti-factor VIII antibodies in hemophilic patients, autoantibodies against endothelial cells in thrombotic thrombocytopenic purpura/hemolytic uremic syndrome as well as in neurological disorders (myasthenia and Guillain-Barre syndrome) [48].

Although most of the reports regarding the use of immunoadsorption are non-controlled studies performed in small groups of patients, now some results from prospective randomized trials are also available. Such studies were performed mainly in rheumatoid arthritis patients. Furst and colleagues compared the results of treatment with sham procedure (apheresis only, with blood circulating through a by-pass loop around the column to ensure blindness) and staphylococcal protein A immunoadsorption in patients with severe rheumatoid arthritis. The significant improvement in clinical symptoms was found in 41.7% of IA patients who completed the whole course of therapy versus 15.6% of those on sham treatment. The duration of response after the full course of treatment was very long and lasted 37 +/- 5.3 weeks [49].

Side effects of LDL-apheresis and immunoadsorption

Side effects of LDL-apheresis and immunoadsorption, although significantly less pronounced in comparison to 'classical' PE are still very important. They may be related to several factors. First, they may be related to the extracorporeal circuit, central venous access, plasma separation procedures or anticoagulation; all these conditions are relatively 'universal' for most of extracorporeal blood purification techniques. More specific adverse effects of LDL- apheresis and IA are related to the contact between plasma and the ligand used. In this respect the staphylococcal protein A deserves special attention. The basic biological role of this agent (being the part of bacterial cellular wall) is to activate immune system and stimulate cytokine release. In addition, depending on technology, SPA may be contaminated with other agents, such as enterotoxins [31,48]. Hence, the treatment with IA may be complicated by allergic reactions and the prophylactic use anti-histaminic drug prior to the procedure is highly advisable. These reactions may be mild and transient, mostly limited to skin (urticaria), but also more generalized, for example resulting in hypotension, joint pains and swelling, fever and even convulsions. Relatively rare complication that may occur in patients treated with IA using staphylococcal protein A columns is small vessel vasculitis related to the development of SPA/SPA-antibody immune complexes [50].

There is an extremely interesting study that focused on possible side effects of IA using double blind, randomized approach, performed in rheumatoid arthritis patients and comparing IA with sham treatment. In this study all patients and study personnel were blinded against the treatment used, and the preparation of the extracorporeal circuit was exactly the same. The only difference between the treatment groups was the by-pass of plasma around the IA column in sham-treated patients. Interestingly, the incidence of adverse reactions was equal in both groups and thus – related probably to extracorporeal circuit itself and/or to plasma separation [49].

As in the case of PE, the series of IA procedures may also lead to systemic immunoglobulin depletion and further immunosuppressive state; hence it may be complicated with serious systemic infections [27]. Albumin deficiency may also occur after few procedures [31].

In the opinion of the authors of this review special attention should be paid on hypotension occurring during LDL-apheresis and immunoadsorption. It must be kept in mind that blood pressure lowering in dextran-sulphate columns may be attributed to enhanced bradykinin release, since prekallikrein becomes activated on the contact with this polyanionic membrane. Hence, it is important to remember that treatment with ACE inhibitors should be discontinued prior to the initiation of IA treatment. The unintentional administration of mentioned drugs may result in severe hypotension and 'anaphylactoid' reactions [3,4]. This seems to be the issue of superior importance since the renin-angiotensin-aldosteron system blockade is currently the most important strategy for amelioration of proteinuria and renal failure progression, with an excellent support form a number of prospective and randomized studies. Many of patients being candidates for LDL-apheresis/IA would additionally benefit from the concomitant use need of these renoprotective of cardioprotective drugs and deprivation of this treatment would be very problematic. However, in these cases switching from ACEi to ATII receptor blocking agent is probably safe [1,3].

Future considerations

Reviewing the literature regarding the issue of LDL--apheresis and immunoadsorption we have a strong impression, that these methods are the milestones in the treatment of many autoimmune diseases since the introduction of steroids and cyclophosphamide as immunosuppressive agents. In our opinion encouraging results of many clinical trials should change the use of this method not only as a rescue therapy for patients who do not respond to other conventional treatment. We believe that these techniques should be employed earlier, possibly even as a first-line therapy in patients who display the risk factors for poor prognosis and fast progression of the disease as well as in those with contraindications for 'classical' immunosupression. We also think, that despite the fact that LDL-apheresis and IA are very expensive, their early use in well-selected cases may also

benefit from financial point of view. An early response to this treatment may significantly reduce the costs of hospitalization, cease unsuccessful immunosupression, limit its complications and possibly reduce overall costs postponing renal replacement therapies.

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The nephrologist's role in the oncology – haematology ward

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Abstract

Cancer patients require care by a multidisciplinary medical team. Although nephrology usually is not the "core" speciality in such a multidisciplinary team, nonetheless it may substantially improve the quality of care. This paper reviews nephrologist's role in the management of the cancer patient.

Key words: cancer, multidisciplinary care, nephrologist.

Introduction

Cancer patients require care by a multidisciplinary medical team. Although nephrology is usually not the "core" speciality in such a multidisciplinary team, nonetheless it may substantially improve the quality of care.

Malignancy and its treatment modalities may be associated with a variety of renal and metabolic abnormalities. Nephrologist is usually called in when one of the following problems is encountered:

- Kidney insufficiency either preexisting or developing in the course of disease
- Glomerular injury presenting as nephritic or nephrotic syndromes
- Obstructive nephropathy causing kidney failure
- Tubulointerstitial disease
- Tumor invasion of the kidney
- Fluid and electrolyte disorders
- Decision regarding renal replacement therapies.

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These may be either the result of the presence of malignancy (local or remote effects) or be associated with treatment or diagnostic procedures.

Acute renal failure (ARF)

May arise as a consequence of the most disorders described in this presentation including acute tubular necrosis, glomerular disease, tubulointerstitial disease, abnormalities of intrarenal blood vessels. Particular attention should be paid to the nephrotoxicity of drugs used in the treatment of cancer patients (*Tab. 3*). In the management of ARF in the setting of oncology-hematology department meticulous attention should be paid to the prevention of ARF. Dialysis may be necessary in more severe cases.

In cancer patients decrease in effective circulating blood volume leading to renal hypoperfusion with consequent decrease in GFR, may be induced by several mechanisms. Most common causes of hypovolemia include vomiting, diarrhoea, edema, hepatorenal syndrome and treatment with interleukin-2 (which induces alterations in vascular permeability, leading to a capillary leak syndrome). Management of prerenal failure is directed towards the correction of the underlying cause and, restoration of extracellular fluid volume to optimal.

Chronic renal failure (CRF)

May be the consequence of most of the disorders described in this presentation including glomerular disease, tubulointerstitial abnormalities, renovascular disease, and chronic obstructive uropathy

Glomerular disease

Glomerular disease is a recognised complication of malignancy. Its true incidence is unknown although it is probably not as frequent as generally thought and does not warrant an extensive workup in search for malignancy. Most frequent glomerulopathies seen in the association with malignancy are membranous nephropathy, minimal change disease, membranoproliferative GN, RPGN, focal segmental glomerulosclerosis

Table 1. **Most common causes of chronic renal failure in cancer patients**

Clinical syndrome	Type of disease
Glomerular disease	Glomerulonephritis Amyloidosis Primary renal cancer Chemotherapeutic drugs
Tubulointerstitial abnormalities	Monoclonal immunoglobulin deposition disease Light chains deposition disease Infiltration by tumor cells Radiation nephropathy Chemotherapeutic drugs
Renovascular disease	Thrombotic microangiopathy (HUS, TTP) Hypertension Renal vein thrombosis
Chronic obstruction	Ureteral obstruction Unilateral obstruction in the case of a single functioning kidney Retroperitoneal fibrosis (irradiation, drugs, carcinoid tumors, reactions to metastases obstruction of the urethra)

and amyloidosis. They were described usually in the setting of Hodgkin lymphoma; other lymphoproliferative disorders; squamous cell carcinoma; adenocarcinomas of the lung, kidney, thyroid, cervix, and gastrointestinal tract [1-5]. This topic is described in more detail elsewhere in this issue of the journal.

Tubulointerstitial disease

Most common forms of tubulointerstitial disease observed in cancer patients are listed in *Tab. 2*.

Obstructive uropathy

May be caused by bilateral ureteral obstruction (or unilateral obstruction in the case of a single functioning kidney) by tumors growing in the vicinity of the urinary tract, or develop as a result of blood clots or stones induced by therapy. Most common tumors causing urinary tract obstruction include:

- bladder tumors and tumors of the collecting systems
- uterine tumors, especially carcinoma of the cervix
- retroperitoneal tumors (rare)
- primary renal tumors (rare).

Other rare causes include: retroperitoneal fibrosis (irradiation, drugs (busulfan), carcinoid tumors, reactions to metastases), or obstruction of the urethra.

Diagnosis is usually made by ultrasonography although it should be remembered that normal-appearing collecting system does not exclude the diagnosis. Treatment is directed toward relieving obstruction by urological procedures or chemotherapy in order to decrease tumor mass [6].

Tumor invasion of the kidney

It is needless to say that primary renal tumors (like renal cell carcinoma) invade renal parenchyma, but usually do not require nephrological consultation. Kidney failure develops only when there is extensive bilateral renal involvement. More commonly kidney insufficiency results from surgery, and consultation may

Table 2. **Tubulointerstitial disease in cancer patients**

Cause	Symptoms
Products or metabolites of cancer cells	
Lysozyme	Hypokalemia Fanconi's syndrome
Immunoglobulin light chains	Renal tubular acidosis Fanconi's syndrome
Hypercalcemia	Polyuria, polydypsia
Amyloid deposits in collecting ducts	Nephrogenic diabetes insipidus
Cast nephropathy in multiple myeloma	Nephrogenic diabetes insipidus
Drugs	
Cisplatin	Hypomagnesemia
Cyclophosphamide	SIADH
Ifosfamide	Fanconi's syndrome
Streptozotocin	Renal tubular acidosis Hypophosphatemia Fanconi's syndrome
Vincristine	SIADH
Change in hormones	
↑ PTH-like	Hypercalcemia Hypophosphatemia
↑ ADH	Hyponatremia (SIADH)
↓ ADH	Hypernatremia (central diabetes insipidus)
Adrenocortical excess	Hypokalemia
Adrenocortical insufficiency	Hyperkalemia

Table 3. **Drugs used in oncology associated with kidney damage**

Syndrome	Drug
Nephrotic syndrome	Mitomycin C Gemcitabine Interferon-2
Acute tubular necrosis	Antibiotics – aminoglycosides, amphotericin, pentamidine, cephalosporin (rare), vancomycin (rare), acyclovir, gancyclovir, foscarnet Chemotherapeutics – methotrexate, cisplatin, carboplatin, streptozocin, semustine, carmustine, ifosfamide, interferon-A, mithramycin
Acute interstitial nephritis	Penicillins, cephalosporins, sulfonamides, thiazide diuretics, loop diuretics, antituberculous drugs, NSAIDs
Chronic renal failure	Cisplatin, semustine, carmustine, streptozotocin, cyclosporine, gemcitabine, and deoxycoformycin
Hemolytic-uremic syndrome	Mitomycin (potentiated by tamoxifen), bleomycin + cisplatin, radiation + high dose cyclophosphamide
Tubular interstitial fibrosis	Cisplatin, carboplatin, cyclosporine, FK-506, ifosfamide
Fanconi's syndrome	Ifosfamide

be asked by surgeon to aid in management plan after tumor removal [7].

Many tumors metastasise to kidneys but usually late in the course of the disease and rarely require nephrological intervention.

Most often kidneys are involved in lymphoproliferative malignancies (acute lymphoblastic leukemia and lymphomas). The spread of malignancy to kidneys is usually manifested by proteinuria, hematuria, increased kidney size in imaging studies and impaired renal function. Treatment includes chemotherapy and local irradiation.

Involvement of kidney in hematological malignancies

Kidney failure is present in up to 20-40% of patients with multiple myeloma at the time of presentation [8-10]. A variety of renal disease is associated with multiple myeloma and might present either as acute or chronic renal failure. Most common pathomechanism is due to the overproduction of immunoglobulin light chains. These include myeloma kidney (which refers to renal failure resulting from filtration of light chains, that lead to a formation of intratubular casts and are toxic to tubular cells), light chain deposition disease, primary amyloidosis, and tubulopathies.

Other renal problems observed in multiple myeloma are hypercalcemia, radiocontrast nephropathy, acute urate nephropathy, direct invasion of the kidneys by neoplastic cells and cryoglobulinemia [10].

This topics is the subject of a separate presentation during present symposium and therefore will not be covered in detail.

Drugs used in cancer patients

Both chemotherapeutics used to treat malignancy and other drugs commonly used to treat complications may induce renal damage. *Tab. 3* shows typical clinical syndromes seen with different drugs

Tumor lysis syndrome

Is characterized by electrolyte abnormalities and frequently acute renal failure. It usually occurs in patients with lymphoproliferative malignancies, most often after initiation of treatment, when massive lysis of tumor cell generates large amounts of uric acid, potassium, and phosphate. Tubular obstruction by uric acid and calcium phosphate crystals may cause acute renal failure. Treatment includes intravenous hydration; alkalinization of urine; use of allopurinol or recombinant urate oxidase; lowering serum potassium levels; and dialysis if necessary [11].

Radiation nephritis

Usually develops 6 to 12 months after kidneys receive dosesgreater than 2000cGy most often in patients receiving whole abdominal radiation therapy or in the bone marrow transplantation setting [12,13]. It manifests with impaired renal function, hypertension, and often with hematuria, oliguria, fatigue, and renal atrophy. Chronic radiation nephropathy develops 10 to 15 years after radiation treatments, and present as chronic interstitial nephritis. Urinary sediment is usually bland. Treatment of radiation nephropathy is limited to the control of elevated blood pressure [13].

Renovascular disease

Thrombotic microangiopathies (TTP and HUS) are closely related disorders presenting as a triad of ARF, thrombocytopenia and hemolytic anemia. In patient with malignancy they may be caused by some types of tumors or by drugs used in chemotherapy [14]. Treatment is directed to the removal of culprit and occasionally plasmapheresis is necessary [14].

Renal venous thrombosis in a cancer patient may be a consequence of tumor growth, hypercoagulability, nephritic syndrome or massive hyperleukocytosis [15,16].

Fluid and electrolyte abnormalities

Most common fluid and electrolyte abnormalities include hyponatremia, hyperkalemia, hypercalcemia and polyuria but almost any electrolyte abnormality may be encountered in cancer patient. Electrolyte abnormalities may be associated with hormonal changes induced by neoplasia (*Tab. 2*), tumor lysis syndrome, treatment (*Tab. 3*) or spread of cancer to bones.

Bone marrow transplantation

Occasionally nephrologist might be called to consult a bone marrow transplant (BMT) recipient. The spectrum of renal involvement in such a patient encompasses most of the aforementioned diseases. Most frequent are ARF, CRF, thrombotic microangiopathies (TTP, HUS) and hepatic veno-oclusive disease (VOD).

Acute deterioration in renal function (defined as doubling serum creatinine) develops in about 50% of the patients after successful BMT, usually as results of sepsis, urinary tract infection, nephrotoxic drugs or in association with VOD [17,18]. VOD usually develops 2 to 3 weeks after BMT and its clinical picture resembles that of hepato-renal syndrome.

Similarly to recipients of solid organs, BMT patients may develop nephrotoxicity associated with calcineurin inhibitors therapy (cyclosporine, tacrolimus), although chronic calcineurin inhibitors nephrotoxicity is less frequent, because these drugs are given in full doses to stable patients for shorter periods of time, than in solid organ recipients. This does not hold true for cases when graft-versus-host disease develops. Late-onset renal failure occurs in up to 20% of survivors of BMT as a consequence of the so-called bone marrow transplant nephropathy [12].

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Lipid disturbances in chronic renal failure – patomechanisms and treatment

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Abstract

Lipid disturbances are a constant feature of chronic renal failure (CRF). They compose a significant risk factor for vascular complications, leading to increased morbidity and mortality in this patients group. The major lipid abnormality in the course of CRF is hypertriglyceridemia, but increased cholesterol level is also common. Numerous studies, including these from our Centre, point to the conclusion that hypertriglyceridemia is a consequence of both, increased TG production and impaired TG removal. In contrast hypercholesterolemia is mainly due to enhanced cholesterol biosynthesis. HMG-CoA reductase inhibitors (statins) compose the most promising group of drugs to treat lipid abnormalities in CRF. Apart from their lipid-lowering abilities they posses non-lipid, so called pleiotropic activities, which make them especially useful in proliferative and inflammatory kidney diseases.

Key words: chronic renal failure, lipid disturbances, fibrates, statins.

Cardiovascular and cerebrovascular complications are as much as 20 times more frequent in patients suffering from chronic renal failure (CRF), when compared to general population [1]. This is mainly due to accelerated atherosclerosis processes in this group of patients. Numerous risk factors for the development of atherosclerosis are present in patients with CRF. Among them lipid disturbances play a particularly important role. Described for the first time almost hundred years ago by Blackall et al. they were predicted much earlier by Richard Bright [2]. They consist mainly of hypertriglyceridemia [3]. This abnormality occurs in as much as 60% of CRF patients. Triglycerides (TG) content is increased in all atherogenic lipoprotein fractions (VLDL, IDL, LDL), and seems to be related to the progression of the disease. Hypercholesterolemia is less common, as it affects 20-30% of patients with renal insufficiency. This percentage is similar to the one observed in general population. However, even in cases of normal total cholesterol level shifts among particular lipoprotein fractions are often observed with a tendency to higher cholesterol concentrations in atherogenic VLDL and LDL, and lower in HDL fraction.

Lipoprotein disturbances in the course of CRF vary significantly depending on the stage of the disease and the method of treatment. On the basis of data obtained from the literature as well as the studies performed in our Department it can be stated that the abnormalities in lipid profile are still present in the course of renal replacement therapy. In hemodialysed (HD) patients they consist mainly of increased plasma TG levels and reduced plasma HDL cholesterol [4]. It is noteworthy that erythropoietin treatment has no impact on lipid profile in HD patients [5]. Impaired lipid metabolism is also common in patients on peritoneal dialysis (PD). Increased concentrations of TG, total and LDL cholesterol, decreased HDL cholesterol level are constant features of lipid profile in PD patients [6]. Probably, to some extent it is a consequence of increased absorption of glucose from the dialysate. But it is after renal transplantation when most severe changes in lipid metabolism occur. Our studies, among others indicate that cholesterol level begins to rise immediately after transplantation reaching the maximum after 2-3 months post transplant [7-9]. In the third month after transplantation total cholesterol level is approximately 20% higher, than pre-transplant. Cholesterol concentration is increased in all the lipoprotein fractions. In

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the course of further treatment the level of total cholesterol decreases, still, after six months after the transplantation it exceeds 200 mg/dl in 80% of patients. It is now well acknowledged that the mentioned above complications are caused by the immunosuppressive treatment, especially the use of cyclosporine and steroids [9].

Despite numerous studies performed in several nephrology centres all over the world the mechanisms of lipid disturbances in the course of CRF are still poorly understood.

Hypertriglyceridemia is the major lipid abnormality in CRF patients. It is believed that this disturbance is mainly a consequence if impaired TG removal. Studies by Bagdade et al. [3,10] demonstrated decreased activities of both lipoprotein and hepatic lipases. The impact of lipogenesis disturbances on hypertriglyceridemia was evaluated in our Centre on a rat model of chronic renal insufficiency. Experimental CRF was induced by our own modification of 5/6 nephrectomy model [11]. First studies, performed 25 years ago showed augmented lipid production in white adipose tissue of CRF animals [12]. This was most probably due to the observed increase in free fatty acid synthase activity. The studies on lipogenesis in experimental CRF were continued recently [13]. They confirmed that the activity of free fatty acid synthase activity was increased in CRF rats. Further, increase in the enzyme's mRNA level and protein abundance was demonstrated, both in liver and adipose tissue of CRF animals when compared to control group. Moreover, increased activities of malic enzyme, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were observed. As these enzymes play a significant part in lipogenesis processes, namely NADPH production, obtained results further support the role of increased lipid synthesis in development of hypertriglyceridemia in the course of CRF. The factors responsible for the stimulation of gene expression of enzymes engaged in lipogenesis include hyperinsulinemia and low leptin concentration. Indeed, results of our earlier studies demonstrate that leptin mRNA level is decreased by 50% in adipose tissue of CRF animals, while leptin is known to inhibit fatty acid synthase gene expression [14,15].

Hypercholesterolemia affects 20-30% of CRF patients. Though not as common as hypertriglyceridemia it is as dangerous, since it is acknowledged that hypercholesterolemia constitutes an independent risk factor for atherosclerosis development [1]. As atherosclerosis processes are enhanced in CRF population, leading to increased morbidity and mortality in this patient group, mechanisms leading to hypercholesterolemia deserve thorough evaluation.

Both in vitro and in vivo studies conducted in our Centre showed a significant impact of enhanced cholesterol biosynthesis on hypercholesterolemia in experimental CRF. The first set of experiments with the use of tritated water injected intraperitoneally proved that the rate of cholesterologenesis was increased both in liver and intestines of CRF animals [16]. Further studies evaluated in vitro incorporation of radioactive cholesterol substrates: [^{14}C]-acetate and [^{3}H]-mevalonate into liver cholesterol fraction [16]. Significantly higher activities of both 'compounds' incorporation were found in livers of CRF rats, when compared to control animals.

Since 3-hydroxy-3-methylglutaryl coenzymeA (HMG-CoA) reductase is the rate limiting enzyme in cholesterol biosynthesis pathway one can suppose that enhanced cholesterologenesis observed in our studies is a consequence of increased HMG-CoA reductase activity. This activity, as well as the whole cholesterologenesis process undergoes a significant diurnal rhythm. It is preserved in the course of experimental CRF, as demonstrated by studies from our Centre [16]. Our experiments on HMG-CoA reductase were, therefore, performed with regard to these diurnal changes in enzyme's activity. We found that liver microsomal HMG-CoA reductase specific activity in CRF rats was increased both during the day and in the night [17]. Similarly, values of HMG-CoA reductase mRNA level were higher in CRF group when compared to control animals.

Patients, as well as laboratory animals with CRF exhibit higher plasma mevalonate concentrations with a concomitant decrease in its urinary excretion [18]. Mevalonate is a direct product of HMG-CoA reductase activity. Therefore, in the presence of elevated mevalonate level HMG-CoA reductase activity and its mRNA level would be expected to be suppressed rather than stimulated. As it is not the case, it seems obvious that normal feedback regulation of HMG-CoA reductase is altered in CRF animals.

The etiology of the described disturbances is poorly understood. It has been suggested that CRF generates an undefined stimulus for HMG-CoA reductase activity in the liver. Feingold and Grunefeld [19] demonstrated that intravenous administration of Tumor Necrosis Factor-alpha (TNF-α) stimulated hepatic sterol synthesis and increased HMG-CoA reductase activity in livers of rats. It is noteworthy that plasma concentrations of TNF-α as well as soluble TNF-α receptors have been found to be increased in chronically uremic patients [20]. On the other hand, however, in the work by Feingold and Grunefeld stimulation of sterologenesis by TNF-α was specifically localized to the liver. Administration of TNF-α did not stimulate cholesterol biosynthesis in the small intestine, adipose tissue, muscle nor skin, while our results indicated that in experimental CRF cholesterolegenesis was stimulated both in liver and intestinal tissue [16]. Therefore, it appears that TNF-α might by just one of many factors responsible for the increase in HMG-CoA gene expression in CRF.

Taking into consideration unfavorable impact of lipid disturbances on CRF patient survival it seems advisable that they should be adequately treated. Dietary guidelines state that total calorie intake should be chosen to attain/maintain a desirable body weight and to prevent weight gain, daily total fat intake should not exceed 35% of total calories: (a) saturated fats < 7% of total calories, (b) polyunsaturated fat up to 10% of total calories, and (c) monounsaturated fat up to 20% of total calories, daily carbohydrate intake should range between 50 and 60% of total calories (predominantly from foods rich in complex carbohydrates, including grains, fruits, and vegetables), and daily protein intake is expected to be about 15% of total calories [21]. But dietary restrictions are usually insufficient in correcting lipid profile, and pharmacological treatment is often indispensable. Fibrates are often introduced, especially in patients with predominant hypertriglyceridemia. How fibrates exert their hipolipidemic action is still unclear. However, it is known that they act as peroxisome proliferator-activated receptor alpha

(PPAR-α) agonists [22]. By activating these nuclear transcription factors they have an impact on free-fatty acid transport. Further, they enhance β-oxidation process, increase the activity of lipoprotein lipase and the expression of apolipoproteins: apoA1 and apoA2, thereby increasing HDL production. Finally, they decrease VCAM-1 and interleukine-6 synthesis, and, therefore, act as anti-inflammatory agents [23,24].

But the most potent drugs lowering plasma lipid concentrations are HMG-CoA reductase inhibitors, i.e. statins. They inhibit endogenous cholesterol synthesis on the level of HMG-CoA reductase. Statins bind to this enzyme at nanomolar concentrations, whereas the natural substrate, HMG-CoA binds at micromolar concentrations [25]. This leads to competitive displacement of HMG-CoA and inhibition of the synthesis of cholesterol precursor, mevalonate. Active cholesterol synthesis is a requirement for VLDL assembly and secretion [26]. As VLDL are precursors of IDL and LDL, statins, by inhibiting cholesterol synthesis decrease the concentrations of all these lipoproteins. Further, reduction of intracellular cholesterol concentration by statins leads to activation of compensating mechanisms to assure intracellular lipid homeostasis. Namely, it activates membrane-bound transcription factors called sterol regulatory element binding proteins (SREBPs) that regulate multiple genes involved in cholesterol homeostasis [27]. SREBPs directly stimulate the expression of more than 30 genes dedicated to the synthesis and uptake of cholesterol, fatty acids and TG, among them the LDL-receptor gene. This leads to an increase in the number of LDL-receptors and intensification of LDL clearance from the plasma providing yet another mechanism, by which statins decrease plasma cholesterol concentration [26]. Lipid lowering properties of statins do not restrict themselves solely to cholesterol. More potent drugs, e.g. atorvastatin or rosuvastatin have been shown to decrease TG levels by 20-30% [28,29].

Beneficial effects of statins in decreasing the risk of coronary complications and prolonging survival have been documented in many large trials, with thousands of patients participating [30-33]. Statins appear also to have a positive impact on the prevention of other vascular complications, e.g. strokes [34]. Similar results have been obtained in patients with kidney diseases, both in persons with mild chronic renal insufficiency, as well as in end-stage renal disease (ESRD) patients [35,36].

Potential usefulness of statins in treating kidney diseases has been investigated by several authors. Kasiske et al. [37] introduced lovastatin in rats with experimental CRF. After 10 weeks of therapy urine albumin excretion was significantly reduced, as compared to the control group. Moreover, the percent of glomeruli with focal glomerulosclerosis was 3 times smaller in the group treated with the statin. In an experiment by O'Donnell et al. [38], after 22 weeks of treating rats with experimental CRF with lovastatin the animals had a 76% reduction in urine albumin excretion and one-sixth the incidence of focal glomerulosclerosis, compared with the vehicle-treated control rats. Other studies dealing with the use of lovastatin in rats with experimental CRF showed decrease in blood urea concentration, mesangial cell proliferation, and glomeruli sclerosis [39,40]. Clinical observations provided similarly promising results. Shoji et al. [41] introduced pravastatin in patients with microalbuminuria in the course of diabetic nephropathy. After three months of such treatment they observed decrease in albumin excretion in all patients. In the work by Rabelink et al. [42] partial remission of nephrotic syndrome was achieved in all patients after 48 weeks of treatment with simvastatin. However, patient groups were small in these studies.

There is also some clinical evidence that statins may slow down the progression of renal insufficiency [43]. These observations gain more importance in the light of the hypothesis that mechanisms leading to glomerulosclerosis are very similar to those responsible for the development of atherosclerosis [44].

Knowing that lipoprotein disturbances lead to atherosclerosis, and given the information that they can also have an injurious impact on kidneys, it would seem self-evident that the mentioned above beneficial effects of statins are related solely to their lipid lowering properties. However, analysis of large clinical trials shows that the advantages of statin use are not associated with the degree of cholesterol reduction [30-32]. In fact, they exceed it by far. Furthermore, a growing number of experimental studies indicates that many actions of HMG-CoA reductase inhibitors are independent from cholesterol lowering. For instance, Hidaka et al. [45] observed inhibition of the migration of cultured porcine smooth muscle cells stimulated with PDGF by simvastatin. This effect was reversed by the addition of mevalonate to the culture. However, supplementation with LDL was unable to reverse the effect of statin. In another work vascular smooth muscle cell proliferation was inhibited by lovastatin [46]. Addition of mevalonate completely reversed this effect, whereas supplementation with cholesterol had no impact on lovastatin induced action. Similar results were obtained in rat mesangial cells, where exogenous LDL did not prevent lovastatin inhibition of cell proliferation [47]. These investigations, as well as many others, show clearly that some effects of statin use are not related to cholesterol lowering.

It is known, however, that by inhibiting HMG-CoA reductase activity statins influence the biosynthesis of more compounds than merely cholesterol.

Mevalonate is the direct product of HMG-CoA reductase activity. Not all mevalonate molecules, however, participate in cholesterol formation. Some of them join together to form 15-carbon, and 20-carbon compunds – isoprenoids, named farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), respectively (*Fig. 1*). These two compounds play a major role in a process called protein prenylation [48]. It consists in post-translational modification of certain proteins by isoprenoids, leading to protein activation. Both, FPP and GGPP have the ability of covalent addition to cysteine residues at the carboxy-terminus of the proteins. The process is mediated by two enzymes, farnesyl transferase and geranylgeranyl transferase. The enzymes recognize a specific sequence at the carboxy-terminus, named CAAX motif, where C means cysteine, A an aliphatic amino acid, and X the carboxy-terminal amino acid, which determines which isoprenoid is to be added. In situations where X is methionine or serine, preferential protein prenylation occurs via farnesyl transferase, and where X is leucine, GGPP addition occurs. The enzymes

Figure 1. **Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) synthesis**

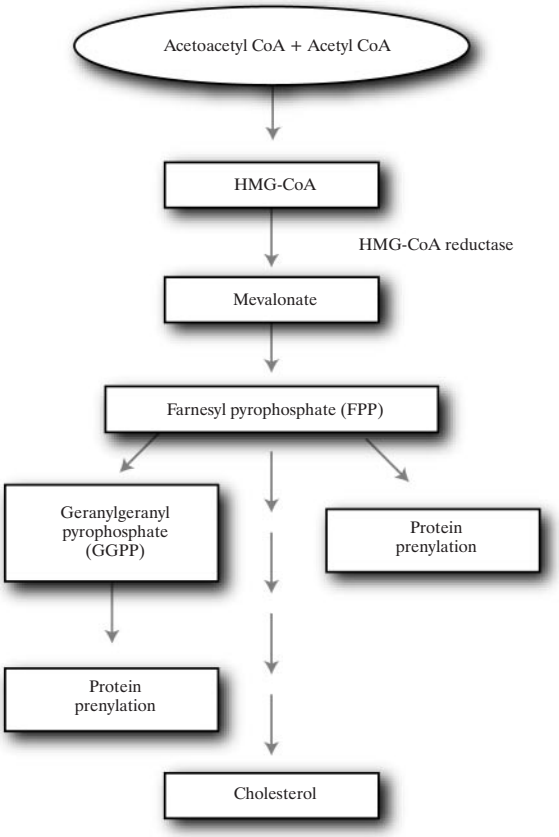
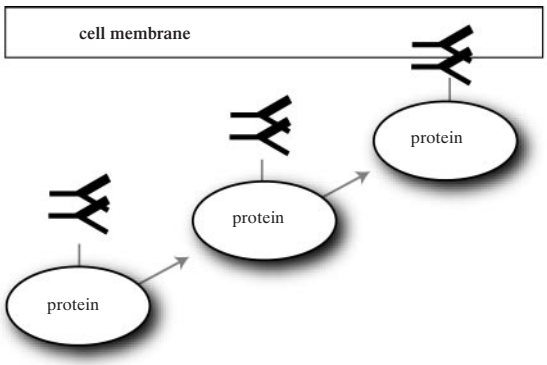


Figure 2. **Prenylation process. Isoprenoid (FPP or GGPP) is attached to the CAAX motif of a protein. This enables attachment of the protein to the inner cell membrane, and protein's activation**



catalyze the attachment of isoprenoid to the cysteine residue of the CAAX motif, and the –AAX moiety is removed. It is now known that prenylation is not confined to proteins containing the CAAX motif. Still, in such cases isoprenoids are also attached to cysteine residue at, or very near the carboxy-terminus of the protein. Prenylated protein is translocated to the inner cell membrane or plasma membrane, where isoprenoid serves as an "anchor" to fix the protein on the membrane's surface, thereby activating it [48] (*Fig. 2*). About 0.5% of the proteins in animals cells are activated through prenylation [49]. Some of them, as Ras or rho proteins are indispensable for cell growth and proliferation [50,51]. Therapeutic strategies that aim at prenylation inhibition can, therefore, have a crucial impact on inhibiting cell growth and proliferation, for instance in primary glomerulonephritis. Results of numerous studies demonstrate that the antiproliferative activity of statins is related, at least in part to inhibition of protein prenylation [51-53].

Cell proliferation is a part of a general inflammatory process which affects kidneys in response to injurious factors. This process, apart from cellular proliferation includes: release of several adhesion molecules, chemokines, pro-inflammatory cytokines, activation of inflammatory enzymes, oxidative stress, alterations in nitric oxide (NO) synthesis, coagulation disturbances etc. One of the markers of the ongoing inflammation is increased plasma concentration of C-reactive protein (CRP). It has also been demonstrated that levels of CRP are associated with increased risk of myocardial infarction and stroke among apparently healthy individuals [54]. The impact of pravastatin on CRP level was evaluated in a group of patients taking part in Cholesterol and Recurrent Events (CARE) trial [55]. After five years patients on pravastatin treatment had median CRP levels 21.6% lower than those on placebo ($p = 0.007$). This effect was, at least to some extent, independent from cholesterol lowering, as the magnitude of CRP reduction associated with pravastatin use did not correlate with the magnitude of LDL reduction. In an interesting study by Sirken et al. [55] statin therapy was demonstrated to decrease erythropoietin (EPO) requirements in hemodialysed patients. Increased levels of CRP have been associated with relative EPO resistance in dialysis patients. Therefore, the authors suggest that the improvement in EPO responsiveness may be caused by the effect of statins on CRP. These observations raise the possibility that HMG-CoA reductase inhibitors may have clinically important anti-inflammatory actions.

Despite potential benefits, statin treatment in CRF patients is still related to many fears and controversies. Numerous reports on myopathy, including rhabdomyolysis after statin treatment are available in the literature [56-58]. This is especially true for patients after renal transplantation, since some statins posses the same path of metabolism with immunosuppressive agents, mainly with cyclosporine and tacrolimus [59,60]. It has to be stressed, however, that statins are safe, when administered in adequate doses. Their ability to lower cholesterol level, as well as their pleiotropic abilities make statins a group of drugs worth considering in CRF patients.

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Acute renal failure: the new perspectives

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Abstract

This review updates the progress which has been made in the recent years in the field of diagnosis, prevention and treatment of acute renal failure (ARF). Despite the better recognition of the etiology and risk factors of ARF this disease is still characterized by high mortality especially when developing in patients with multi-organ failure. The origin of ARF is clearly multifactorial but its pathomechanism shows many similarities regardless of a cause. Due to the latest achievements in the understanding of the pathogenesis of ARF better preventive and therapeutic strategies are being developed. Some of them have been successfully tested in experimental settings. Surprisingly most of the human studies showed that preventive methods other than simple hydration could not change the course of the disease or even could be harmful. Also the critical issue remains early ARF detection. In turn in cases of fully developed acute tubular necrosis the major concern is to prevent secondary complications and organ failure and to introduce appropriate dialysis therapy. Although not yet supported by a significant reduction in mortality in most severe cases of ARF some progress has been made due to the development of new convective intermittent or continuous renal replacement therapies, notably hemodiafiltration.

Key words: acute renal failure, renal replacement therapy, hemodialysis.

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Introduction

Acute renal failure (ARF) is a common clinical condition that affects as much as 5-7% of all hospitalized patients. Although prevalence rate of ARF substantially decreased in some clinical situations, e.g. after surgical intervention or delivery, surprisingly no significant general improvement in mortality has been recorded [1,2]. Overall this clinical entity still carries a significant mortality rate of 20-70%. The problem of ARF is of major concern especially in cases complicated by non-renal organ failure. The presence of sepsis and associated multi-organ failure results in the highest mortality. Although in mild cases of ARF an average mortality rate is 20% those requiring dialysis have mortality rates of about 40-50% and in the intensive care units patients with ARF carry the highest mortality rate reaching 70% [2]. It is interesting that during the past 50 years these rates remained unchanged mainly because more and more patients developing ARF are older and have multiple co-morbidities [2]. Most of the preventive strategies which appeared promising only several years ago, e.g. loop or osmotic diuretic agents, atrial natriuretic peptide, dopamine, endothelin receptor antagonists, have been found unsuccessful and therefore further studies in this field are mandatory. In contrast some progress has been achieved due to an introduction of new renal replacement modalities which appear to be especially suitable for the treatment of severe cases of ARF. Unfortunately, human studies which have been carried out to date are scarce and the results are conflicting.

Pathophysiology of ARF

Presently it is evident that either the sudden or persistent changes in glomerular filtration, tubular dysfunction or renal perfusion may result in ARF. The knowledge of the pathomechanism is crucial with respect to the development of preventive measures and therapeutic interventions. According to newly proposed classification not three (initiation, maintenance and

recovery) but four phases (initiation, extension, maintenance and recovery) of ARF have been recognized [1]. The initiation phase with nonspecific hemodynamic changes may evolve into the extension phase which is characterized by alterations in renal perfusion leading to hypoxia. In the next phase an inflammatory response predominates resulting in epithelial and endothelial injury which occurs mainly in the cortico-medullary part of a kidney.

ARF is the complex, dynamic process involving renal vasculature and tubules leading to renal injury. The first stage is the reduction of regional renal blood flow which initiates the kind of a chain reaction leading to ARF. Vasoconstriction begins as a result of altered vascular reactivity associated with the endothelial damage and inhibition of the nitric oxide synthase activity. Reduced arteriolar renal blood flow causes cortical proximal tubules cells damage mainly because of cyclic GMP depletion [3]. Additionally endothelial activation and injury result in cell swelling and loss of potency of endothelial barrier leading to mechanical obstruction and interacting with leukocytes and platelets. Those factors may trigger an expression of adhesion molecules (ICAM-1, MIP-2, KC, and CXC) and activate coagulation pathways. That in turn leads to accumulation and activation of leukocytes and the interplay between leukocytes and endothelium which end with cellular infiltration and inflammatory response with whole spectrum of proinflammatory cytokines release (e.g. IL-1, TNF-α). Resulting neutrophil accumulation leads to complement-mediated injury. Tubular cells are also involved in the initiation of the inflammatory response which is accompanied by a massive production of IL-6, TGF-β, RANTES, MCP-1, ENA-76, Gro-α IL-8, TRAF6 and Fas. The prolonged ischemia may also affect parenchymal cells. Finally all of these events may result in cell death which could occur either by necrosis (typically in the proximal tubules) leading directly to elimination of damaged cells during post-ischemic kidney reperfusion events, or apoptosis or programmed cell death (mediated by the expression of bcl2 family genes, i.e. bax, bak, bad and caspases -3, -6, -7) [3,4].

Definitions of ARF

Till now several different definitions of ARF have been proposed. Most of them used arbitrary defined rise of the serum creatinine concentrations as a major criterion for ARF diagnosis. According to them in order to fulfill the criteria of ARF serum creatinine should rise of more than 50% from the baseline value or to over 3.0 mg% (270 μmol/l). Thus, the current definition of ARF is based on serum creatinine or a volume of urine produced which frequently cannot support adequate decisions for therapeutic intervention. However no widely accepted ARF definition has been proposed. Such definition should ideally show the association between the development of ARF and the risk of mortality or the likelihood of recovery of renal function. Extremely complex definitions [5] are likely impractical for clinical evaluation. Precise quantification of the glomerular filtration rate (GFR) with inulin clearance technique might be useful for research purposes but is impractical, cumbersome and expensive. Serum creatinine concentration changes may not reflect GFR precisely enough to be used for monitoring of kidney function [3]. The heterogeneity of ARF and the lack of widely available markers of renal injury, the need for effective diagnostic and classification scheme are points to reconsider ARF definition.

Classification of ARF

The proper assessment of kidney function should take into account several elements such as a function of glomeruli, endothelium, renal tubules and urinary tract. Not only the severity, type and site of injury but also an individual patient's susceptibility can modify the course of ARF. The vital factors that should be taken into consideration are therefore age, sex, co-morbidities, etiology and nature of ARF (e.g. coexisting oliguria), timing of ARF, the nature of an underlying disease, presence of sepsis and nutritional status. The ARF definition relies neither on the presence of specific markers nor on the severity of signs or symptoms. ARF, with its complexity and subsequent multi-organ effects, should be seen in similar manner to other critical clinical entities like sepsis or acute pancreatitis. When all these factors are taken into account the term "ARF syndrome" should be used [3]. It is easy to compose this with RIFLE (Risk, Injury, Function, Loss, End-stage) criteria suggested by ADQI (Acute Dialysis Quality Initiative) [6]. Mehta et al. has recently proposed a new classification of acute renal failure which could help clinicians to categorize patients with ARF (*Tab. 1*) [3]. This allows individual assessment of a patient according to his clinical stage including preexisting diseases (each element is pointed 1 to 4 in each of the following categories – susceptibility, the response to an insult, and end-organ damage). Although appearing promising this classification needs to be tested in clinical settings. For clinical decision making also some sensitive and specific markers of the severity of renal injury and renal response to injury would be helpful.

New insights into a diagnosis of ARF

A rise of serum creatinine concentration often follows an initiating event of ARF with highly variable delay and therefore cannot be recognized as an early marker of its development. Recently KIM-1 (kidney injury molecule 1) – type 1 transmembrane glycoprotein that is upregulated in post-ischemic regenerating rat kidney has been proposed as an early marker of ARF. The physiologic role of this molecule includes the dedifferentiation of epithelial cells, promotion of proliferation and epithelial-mesenchymal transition. Studies of Ichimura et al. and Han et al. provided a strong evidence that this marker for renal injury is highly specific for tubular destruction [7,8]. KIM-1 appeared to be a very sensitive indicator of toxemic ARF and its expression was enhanced in proximal tubule epithelial cells in biopsy specimens taken from patients suffering from ARF [7]. KIM-1 urinary excretion increases after mild tubular injury even before urinary casts appear, and then returns to baseline when the tubular epithelium regenerates. In addition, increased expression of KIM-1 in the proximal tubule shows a tight correlation

Table 1. **The new classification of acute renal dysfunction (S-I-R-E) [3]**

	Stage			
Domain	**1**	**2**	**3**	**4**
Susceptibility **(S)**	None GRF>90	Known preexisting kidney disease (CKD 2) 60<GFR<89	Known chronic preexisting kidney disease (CKD 3) GFR<60	Known preexisting kidney disease (CKD 2) 60<GFR<89 Presence of one risk factor
Insult **(I)** nature time	Known<24 hours	24<Known<48 hours	Unknown >48 h	Both unknown
Response **(R)** Cr increase mg% GFR decrease %	0.5<increase<1 25<decrease<49	1<increase<2 50<decrease>74	increase>2 decrease>75	increase>3 decrease<10
Nonrenal organ failing (E)	None	Single organ	Two organs	>2 organs

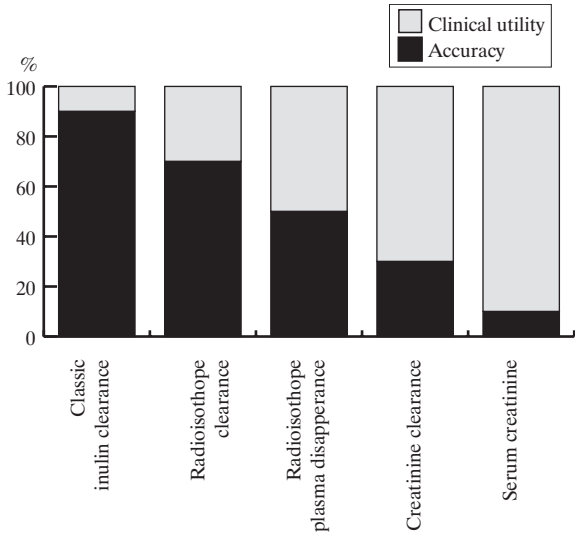
creatinine serum concentration (Cr) – mg%, GFR – ml/min according RIFLE criteria risk factors: diabetes mellitus with microalbuminuria, dehydration, multiple myeloma, congestive heart failure, decompensated cirrhosis organs failing includes: respiratory, CV, neurological, liver, hematological failure

with the development of ARF. Most importantly, no expression of KIM-1 has so far been detected in the normal human kidney [8]. Several other studies tested other suspected early markers of renal injury. Recently another new protein has been discovered. It was named after its chemical structure a cystein-rich protein (CYR 61). CYR 61 could be detected in large quantities in the urine shortly after ischemia reperfusion injury develops [9,10]. Recently, Pilar et al. proposed a new marker asmac/diablo protein (a mitochondrial protein which may block an apoptosis inhibitor IAPs) which may play and important role in regulating amplification of lethal stimuli potential in renal cells during AFR [11]. Although this protein measurement in vivo may be of potential relevance in the detection of early changes in ARF its clinical usefulness has not yet been proven. Still the whole spectrum of biomarkers is being extensively studied in the kidney proteom [12,13]. Another extensively studied protein – cystatin C appears to be a promising marker of changes in GFR, but its serial measurements during the ARF will still require validation in both experimental and clinical conditions [12].

GFR measurements are still of major importance in monitoring the course of ARF. Quantification of GRF could help to modify doses of drugs and more precisely assess the timing of dialysis initiation. An accurate GFR estimation may be also of importance with respect to prognosis. Additionally, GFR assessment could help us to assess the effect of specific therapeutic interventions and provide information on the severity of renal dysfunction (*Fig. 1*). Rapid changes of serum creatinine during ARF are a major limitations to use methods of indirect assessments of GFR which are based on serum creatinine. The most useful is the GFR estimation based sorely on serum concentrations of such markers as inulin or iothalamate. With this method even the rapid changes in GFR could be detected. The new HPLC technique for the measurement of iothalamate concentration has recently been introduced and validated [14]. The use of plasma clearance of radioactive markers (e.g. 51CR-EDTA, 99mTc-DTPA) allows avoiding invasive procedures and also shows an excellent correlation with the GFR measured with standard iothalamate method. In addition those techniques combine estimation of GFR with renal imaging. Modern techniques of GFR estimation should provide more accurate measurements in the case of rapid changes of GFR in the course of ARF [14].

Magnetic resonance imaging is a method which usefulness cannot be underestimated. Whole-body three dimensional MR angiograms may show occlusion or stenosis of the renal arteries as well as kidney diameters or contrast-enhanced images of perfusion changes within renal cortex and medulla. Two new MRI techniques have been extensively studied. Blood oxygenation level-dependent (BOLD) MRI depends on hemoglobin as an endogenous contrast agent according to its paramagnetic (deoxyhemoglobin) and diamagnetic (oxyhemoglobin) capabilities. It provides the non-invasive estimation of tissue pO_2 which reflects medullary hypoxia. Unfortunately with this method one cannot distinguish between alterations of oxygenation caused by perfusion changes or changes in oxygen consumption. Blood pool contrast agents improve high-resolution MRI imaging (allowing better visualization of small vessels) due to reduced interstitial diffusion and prolonged circulation in the blood. This technique detects medullary perfusion defects precisely and may differentiate a cortical from medullary perfusion. Thereby it could offer us the possibility of studying vascular pathology [14]. Recently the micro-MRI methods have been developed in order to measure tubular function and the extent of renal inflammation (the two factors which role in the pathogenesis of ARF may be relevant). Unfortunately, both techniques are in the early stages of experimental research. The first method is based on the use of dendrimer-based contrast agents (also known as the 4^{th} generation agents) and allows to precisely monitoring proximal tubule function after injury [15]. The latter uses negative imaging agent (e.g. dextran-coated ultra small superparamagnetic iron oxide – USPIO) which is quickly internalized after the administration by monocytes/macrophages

Figure 1. **The comparison of the methods used to assess the glomerular filtration rate**



what makes the severity of infiltration and inflammation easy to monitor. Currently USPIO is being used in preclinical studies [16]. The non-invasive examining of patients with ARF using MRI may increase our understanding of the pathomechanism of this disease and likely change the therapeutic regimens in the future.

Two-photon fluorescence microscopy is a method of imaging which allows quantifying apoptotic changes with the near-infrared light. Due to this unique feature this method could be helpful in monitoring of wide spectrum of pathologic conditions including ischemic kidney injury [14].

Some recent experimental studies suggested that ischemia or transient ureteral obstruction may have the protective effect against subsequent ischemia. This protection is associated with a decreased inflammatory response and KIM-1 expression [12].

The new perspectives for therapy

Erythropoietin is a growth agent that has a strong cytoprotective and anti-apoptic properties. Vesey et al. proved that Epo may have an inhibitory role in the regulation of tubular cells apoptosis in vitro and their functional recovery [17]. This concept, although promising, has not been so far supported by clinical studies.

New exciting possibilities are also provided by the studies on stem-cells applications in ARF. Such therapy could give the opportunity to replace damaged kidney structures and a chance for an easiest and an earliest recovery of patients suffering from ARF. Still the great barrier has to be overcome because unlike the heart muscle the kidney consists of at least 26 major types of cells and each of them is highly specialized and differentiated. Thus to achieve the full renal recovery these cell types should be replaced in order to give them a potential to form kidney structures such as glomeruli. Recently with the application of new engineering methods it was shown that the stem-cells could differentiate and form renal tissues [18]. In the recent studies bone marrow stem-cells were found to differentiate in vitro into such kidney structures as tubular epithelium and whole proximal tubules, glomeruli, mesenchymal cells or small vessels [19]. With this technique it was found that hematopoietic stem-cell introduction led to complete renal tubules regeneration after ischemic injury [20]. Unfortunately, the stem-cell science is still at the early stage of development. It has a great potential but also brings huge controversies about embryonic researches and human cloning procedures.

Recently a number of new genes which regulate cell cycle regeneration after renal injury have been identified. Therapeutic interventions involving this gene expression can become most potent and simplest future method of reparation and regeneration of kidney cells [21].

Management strategies

The complexity of causes of ARF and heterogeneity of patients' populations which are at risk of ARF make clinical studies an extremely difficult task. It would therefore be very challenging to propose a uniform management strategy.

Numerous initially promising therapies of ARF based on such agents as loop diuretics or low ("renal") doses of dopamine which could dilate the renal vasculature have been found of no or only of very limited value in clinical conditions [22]. To date volume correction stays the most efficient and evidence-based intervention in most patients with ARF [23]. In the last two years several studies were published which showed that a mucolytic agent with potent antioxidative properties – acetylcysteine could be effective for the prevention of contrast media-induced acute worsening of renal failure. Most of the patients in those studies had chronic renal disease which is a major risk factor of ARF. Seven of those studies which comprised a total number of 805 patients were included in the recent meta-analysis which showed that the administration of acetylcysteine reduced the risk of contrast nephropathy by 56% compared to hydration alone [24]. There is no doubt, however, that fluid balance should be adequately controlled. It is to note that not only dehydration but also excessive hydration should be avoided since Lowell et al. recently reported a strong positive association between fluid overload and mortality, suggesting that fluid management should be carefully observed and limited [25].

The patients in whom serum creatinine concentration increases or oliguria (<400 ml/day) develops are usually consulted by nephrologists. Surprisingly in one study it was found that the timing of nephrologists referral has no significant influence on mortality rate [26], but according to the other analysis patients who were delayed with nephrology consultation had higher non-renal organ failure rates [27].

The natural history, clinical needs, the course of the disease/ /pattern of illness with the predicted therapy response determine and could identify the exact time point of intervention. Each of initial three phases of the aforementioned new ARF classification, may be a target for therapies aimed at prevention, limiting extension or treatment of established ARF, respectively. That helps to characterize the renal response, to institute supportive

therapy (adequate dialyses type) and to stabilize non-renal organ dysfunction. However, each of a new schemes, even the most adequate, has limitations and therefore should be tested to prove its utility.

In severe cases of ARF when there is no chance of early recovery of renal function and serum creatinine rises renal replacement therapy (RRT) should be initiated. Unfortunately, the dose of RRT is often low and inappropriate. The most probable reasons are the presence of high catabolic rate, recirculation of blood and cardiovascular instability that occur frequently in cases of ARF [27]. Despite intensive research leading to a substantial improvement of dialytic procedures especially in intensive care units (timing, duration frequency, dose and modality of RRT) no uniform therapeutic scheme has been established [3]. Although the timing of intervention, the amount and frequency of dialyses may affect outcome, dialysis treatment should be clearly individualized [27]. Continuous renal replacement therapies (CRRT) may show an advantage over intermittent hemodialysis or hemofiltration. It was found that although those methods allows better fluid and metabolic control than standard hemodialysis, it did not increase patient survival rate and decrease the time to renal recovery. Recent studies showed the association between the severity of illness and outcomes after CRRT. Similarly dialyzer membrane biocompatibility or its permeability (low vs high-flux membranes) had no major influence on survival of patients with ARF [28]. There is also no clear advantage of intensive vs less intensive dialysis in ARF although Ronco et al. [29] showed that large volume of ultrafiltration in continuous veno-venous hemofluction (CVVH) improves survival. The retrospective study analyzing the influence of frequency of a dialytic procedure on survival showed that the highest mortality rate was observed in a group of patients who had one dialysis session during the course of the ARF (74.8%), lower for those dialyzed 2-10 times (66.7%), and 10-20 times (50%). If the patients had more than 20 sessions the mortality was even higher (61.5%) [27,29]. No definite conclusions could be drawn from such analyses, however, since the patients who were dialyzed more intensively and longer periods must have had more severe course of the disease.

In patient with end stage renal disease (ESRD) a proper dose of hemodialysis is usually set based on Kt/V calculation and clinical condition. The dose of dialysis prescribed to patients with ARF is much more difficult to establish. Kanagasundarm et al. [30] showed that the calculation of equilibrated Kt/V is also of potential value in patients with ARF. In general, hemodialysis sessions (when using intermittent therapies) should be more frequent in ARF than in patients with ESRD because of the differences in the distribution of urea and invalidity of a steady state assumption in ARF.

Peritoneal dialysis is an acceptable method of treatment of ARF, however, due to technical and practical limitations it is mainly used in small children. In general it could be used in patients without associated severe infections. Since the efficiency of this type of dialysis is generally lower than of hemodialysis, this method should not be used in cases of ARF with multi-organ failure or in case of prominent hypercatabolism [31].

The coupled plasma filtration adsorption (CPFA) is a new and promising method of blood purification which can be used in patients with sepsis and multi-organ failure. This method may be used to efficiently reduce levels of pro- and anti-inflammatory mediators. It also decreases mononuclear cell proliferation [32].

Conclusions

In summary, the knowledge of ARF pathophysiology, definition, classification, prevention and treatment is a dynamic matter. Each year brings new concepts and insights, reveal novel perspectives or management strategies. Despite heterogeneity of ARF patient's population new promising preventive and management strategies are being tested. The reduction in the incidence rate of ARF is seen in the younger patients mainly due to improved preventive methods and awareness of the problem among medical professionals. Still a lot has to be done in the treatment of ARF in the elderly and in patients with multi-organ failure. The reduction in mortality rate in ARF remains a major challenge for nephrologists in the new millennium.

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Endothelial dysfunction, atherosclerosis and thrombosis in uremia – possibilities of intervention

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Abstract

Chronic renal failure is a state of prominent endothelial dysfunction, accelerated atherosclerosis, high incidence of thromboembolic complications and excess cardiovascular mortality. We reviewed up-to-date experimental and clinical data showing close and deleterious links between these entities. Emerging therapeutic interventions aimed at improvement of endothelial function and better clinical outcomes in chronic kidney disease patients were also discussed.

Key words: atherosclerosis, cardiovascular disease, endothelium, thrombosis, uremia.

Introduction

Although uremia is regarded as a clinical model of bleeding diathesis, nowadays a tendency to thrombosis prevails in these patients. Atherosclerosis and thrombotic complications are the main causes of morbidity and mortality in different stages of chronic kidney disease, particularly in patients with renal failure.

In vivo the most important interaction in hemostasis is the one between platelets and vascular endothelium. Platelet function is usually impaired in uremia, although enhanced platelet aggregation in dialyzed patients has been demonstrated [1].

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Antithrombotic function of endothelium

Endothelium produces many substances (*Tab. 1*) with autocrine and paracrine activity [2]. Factors which are regulated by the endothelium and take active part in hemostasis and thrombosis are shown in *Tab. 2*.

One of the most important functions of endothelial cells is the prevention of non-physiological activation of blood coagulation resulting in thrombosis. This activity is mediated by the negative charge of the endothelium provided by surface expression of heparan sulfate proteoglycans and the secretion of prostacyclin, nitric oxide (NO), tissue factor pathway inhibitor (TFPI) and tissue plasminogen activator (t-PA). Endothelial expression of thrombomodulin (TM), which binds to thrombin and activates anticoagulant protein C, plays a very important antithrombotic role. A concentration-dependent role of TM in modulating activity of thrombin activatable fibrinolysis inhibitor (TAFI) is also acknowledged. Endothelial damage triggers thrombus formation due to decreased antithrombotic potential, expression of tissue factor (TF) and release of von Willebrand factor (vWf) as well as plasminogen activator inhibitor (PAI-1). Vascular tone, leukocyte adhesion, permeability to nutrients, macromolecules and leukocytes is also normally regulated by the endothelium. It has become increasingly evident that the peripheral vasculature exhibits striking regional and segmental heterogeneity in the influence of the endothelial cell layer on vascular tone. This heterogeneity arises in part from differences in the endothelial cell microenvironment (autocrine and paracrine substances), and in the influence of hemodynamic forces, such as local pressure and shear stress. Endothelial cells produce also vasoconstrictive substances like endothelin-1 and thromboxane A_2. A delicate balance between endothelium-derived relaxing and contracting factors maintains vascular homeostasis. Upon disruption of this balance by inflammatory and traditional cardiovascular offenders, the vasculature becomes susceptible to atheroma formation. Inflammatory mediators appear to play a fundamental role in the initiation, progression and the eventual rupture of the atherosclerotic plaque.

Table 1. **Autocrine and paracrine substances released from the endothelium (according to Verma et al.)**

Vasodilators	NO, prostacyclin, endothelium-derived hyperpolarizing factor, bradykinin, adrenomedullin, C-natriuretic peptide
Vasoconstrictors	ET-1, angiotensin-II, thromboxane A_2, oxidant radicals, prostaglandin H_2
Antiproliferative	NO, prostacyclin, transforming growth factor-β, heparan sulphate
Proproliferative	ET-1, angiotensin-II, oxidant radicals, platelet-derived growth factor, basic fibroblast growth factor, insulin-like growth factor, interleukins
Antithrombotic	NO, prostacyclin, plasminogen activator, protein C, tissue factor inhibitor
Prothrombotic	ET-1, oxidant radicals, plasminogen-activator inhibitor-1, von Willebrand factor, thromboxane A_2, fibrinogen, tissue factor
Inflammatory markers	CAMs (P- and E-selectin, ICAM, VCAM), chemokines, nuclear factor κ-B
Permeability	Receptor for advanced glycosylation end-products
Angiogenesis	Vascular endothelial growth factor

Table 2. **Regulation of hemostasis and thrombosis by the endothelium (according to Cines et al.)**

	Antithrombotic	**Prothrombotic**
Coagulation protein binding sites	Glycosaminoglycans/ATIII TFPI Thrombomodulin	Binding sites for: fibrin, FIX, IXa, X, Xa, FXII, kallikrein Tissue factor Thrombin receptor Receptor for protein C/APC
Substances produced and/or stored by platelets	PGI_2 NO ADPase	vWF PAF Fibrinogen FV FXI
Fibrinolytic factors	t-PA production u-PA expression u-PAR Plasminogen binding sites Annexin II	PAI-1, PAI-2 PAI-3 (protein C inhibitor) TAFI activation
Vasomotor factors	NO PGI_2	TxA_2 ET-1

Nitric oxide is a particularly important endothelium-derived mediator because of its unique vasodilatory, antiplatelet, antiproliferative, antiadhesive, permeability-decreasing and antiinflammatory properties.

Dysfunction of the endothelium, inflammation and thrombosis

Endothelial dysfunction prevails when endothelial properties have changed in a way that is inappropriate with regard to the preservation of organ function [3]. For example increased vascular tone and permeability may contribute to increased blood pressure and atherogenesis. Current concepts recognize atherosclerosis as a dynamic and progressive disease arising from the combination of endothelial dysfunction and inflammation [4]. Endothelial dysfunction leads to the loss of its antithrombotic and profibrinolytic properties and the development of a prothrombotic and antifibrinolytic status. Such alterations may differ depending on the extent of the injury and intrinsic properties of the endothelium (i.e. venous vs arterial vs microvascular). Endothelial activation designates one specific type of endothelial dysfunction characterized by increased cytokine-induced interactions with blood leukocytes, where adhesion molecules and chemoattractants become essential [3]. Endothelial cell dysfunction can promote transduction of atherogenic risk factors, thus playing an important role in the initiation and progression of atherosclerosis. Oxidatively modified low-density lipoproteins (oxLDL), cholesterol, smoking, hypertension, angiotensin II and diabetes may initiate atherosclerosis through endothelial activation. The predilection of atherosclerosis to arterial branching points is explained by nonlaminar or even turbulent blood flow, which increases shear stress and activates endothelial cells. Endothelial function cannot be measured directly in humans, thus indirect estimates are often used in clinical research, including endothelium-

dependent vasodilation and plasma levels of endothelium-derived substances such as NO, vWf, soluble TM, endothelin, circulating adhesion molecules, t-PA and PAI-1.

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a novel mediator of endothelial cell activation [5]. In addition to being the main receptor for oxLDL, LOX-1 has the ability to bind damaged or apoptotic cells and activated platelets, and reduce the intracellular concentration of NO in endothelial cells [6]. Protease-activated receptors (PARs) are G-protein-coupled receptors which link tissue injury with appropriate cellular responses, such as inflammation and tissue repair, both of which may contribute to disease progression [7]. There are four PARs out of which three (PAR-1, PAR-3, and PAR-4) are activated by thrombin. PARs are expressed by a variety of cells including endothelial cells and platelets. Their signaling impacts the initiation, progression, and complications of atherosclerosis.

Injured vascular endothelium may be repaired thanks to bone marrow-derived endothelial progenitor cells which have an ability to differentiate into functional endothelial cells [8].

Endothelial dysfunction and thrombosis in uremic patients

Growing amount of evidence indicates that endothelial function is disturbed in uremic patients. In line is the fact that forearm artery dilatation is strictly related to the degree of glomerular filtration rate (GFR) [9]. High levels of endothelium-derived regulatory proteins including antifibrinolytic and prothrombotic PAI-1 have also been observed. As PAI-1 is produced not only by endothelial cells but also by hepatocytes, adipocytes and vascular smooth muscle cells, a panel of endothelium derived substances should be determined to validate the assumption of endothelial dysfunction or damage. Several of these molecules are increased in concert in uremia [10]. There is a strong correlation between plasma levels of PAI-1 and the prevalence of cardiovascular disease in uremia [11]. Elevated TF levels as well as increased concentrations of vWf were also described in uremic patients [12-14].

Endothelial dysfunction is also likely to be due to oxidative stress which increases in different stages of chronic kidney disease, including uremia [15-17]. Markers of oxidative stress, endothelial injury, extrinsic coagulation pathway activation and intima-media thickness of the carotid artery were strictly related with the presence of coronary heart disease in hemodialysis (HD) patients [16]. Moreover, oxLDL are cytotoxic for endothelial cells. In hypertensive patients with reduced creatinine clearance plasma fibrinogen, prothrombin fragment 1 + 2 (a marker of thrombin activation) and D dimer (a marker of thrombosis) were elevated. In uremic patients increased blood concentrations of fibrinogen, fibrinopeptide A and thrombin-antithrombin complexes (markers of thrombin activity) were observed [18]. Decreased activity of protein C was demonstrated in patients with renal failure [19]; there is also a report on low levels of free protein S and an active anticoagulant fraction of protein C in these patients [20].

Endothelial dysfunction may play a role in the progression of chronic renal failure by increasing intraglomerular pressure and glomerular basement membrane permeability, as well as indirectly – by influencing mesangial cell and podocyte function in a paracrine fashion. Inflammatory cytokines are also likely to be involved, as uremia can be considered a state of chronic low-grade inflammation.

Thrombosis and atherosclerosis in uremic patients

In uremic patients a striking association of inflammation, high fibrinogen levels and thrombosis was observed [21]. Hypoalbuminemia – a result of malnutrition and/or inflammation, is strongly associated with arteriovenous (AV) graft thrombosis in HD patients [22]. Endothelial cell activation, vascular smooth muscle proliferation and extracellular matrix deposition are also known risk factors for AV graft thrombosis [23]. Assessment of circulating PAI-1 levels can identify patients who are at risk for developing atheromatous cardiovascular disease [11].

Coronary artery thrombosis is generally a result of atherosclerosis, which develops primarily as a result of endothelial damage. Atherosclerosis is a very frequent complication in uremia due to coexistence of hypertension, hyperhomocysteinemia, inflammation, malnutrition and increased oxidative stress. Elevated triglycerides, intermediate-density and very-low-density lipoproteins as well as lipoprotein A, and lowered high-density lipoproteins increase the risk of atherosclerosis in end-stage renal disease [24].

Thrombotic events may also be associated with renal transplantation. Antibody-mediated or cyclosporine-induced endothelial damage may play an important prothrombotic role.

The most common type of thrombosis in uremia results from disruption of the vulnerable atherosclerotic plaque. This is a consequence of exposure to hemodynamic stress. Erosion of the plaque, characterized by areas of endothelial cell desquamation, exposes a prothrombotic surface. Even greater prothrombotic stimulus arises from the rupture of fibrous cap and removal of its contents into the lumen. Subendothelial collagen, TF and vWf become accessible in circulation, promoting coagulation and thrombin formation [4]. Then, platelet activation and aggregation ensue, mediated by interaction with thrombin, TF and vWf. Tissue factor overexpression by endothelial cells and macrophages is enhanced by the presence of inflammatory mediators within the plaque, namely interleukin-1, tumor necrosis factor (TNF) alpha, and CD40/CD40L – members of the TNF family. In response to this vascular insult, thrombogenicity is further favored by the activation of PARs on platelets and in the adjacent tissue [7].

Treatment

Specific interventions which could heal the diseased endothelium in renal failure patients are not available. Smoking cessation, use of ultrapure dialysis fluid and biocompatible membranes for HD procedures, administration of statins,

angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, endothelin blockers, estrogens, L-arginine, antioxidants and folic acid are, however, likely to improve some aspects of endothelial function.

Angiotensin-converting enzyme inhibitors decrease the levels of PAI-1 in plasma, thus promoting fibrinolysis. They also have antioxidative properties and cause a decrease in angiotensin-II and an increase in bradykinin activity. Angiotensin receptor blockers seem to act in a similar way although they do not increase the bradykinin activity.

Reduction of oxidative stress during HD may be reached by the use of biocompatible membranes and ultrapure dialysis fluid. Hypercholesterolemia, hyperhomocysteinemia and smoking are associated with a marked increase of asymmetric dimethylarginine (ADMA) – a known NO synthase inhibitor. Antioxidants, like vitamin C and vitamin E, have been used in uremic patients quite extensively. The lowering of reactive oxygen species in patients treated with vitamin C positively influenced their survival [25]. Nitric oxide is generated in the endothelium from L-arginine by NO synthase. In uremia L-arginine availability may be low [26]. Tetrahydrobiopterin is an essential factor for NO synthase function. When tetrahydrobiopterin is oxidized, the synthesis of NO is compromised and superoxide-peroxynitrite is generated from NO, thus amplifying the oxidative stress [27]. Vitamin C and selenium-containing glutathione peroxidase may reconvert tetrahydrobiopterin to the non-oxidized state, which normalizes NO production. A negative effect of vitamin C on an increase of serum uric acid level in uremia should be taken into account. Selenium supplementation has not been properly controlled. Quite safe may be vitamin E, which can be used orally or as vitamin E-modified cellulose membranes for HD; the clinical results are, however, conflicting and the dialysis cost is substantially increased [28,29]. The placebo-controlled SPACE study showed reduction in myocardial infarction and composite outcome of cardiovascular end-points in HD patients treated with 800 IU of vitamin E daily [30]. Red blood cells contain a high amount of antioxidants – particularly of reduced glutathione peroxidase, so anemia treatment should be included into antioxidative armamentarium. It is also interesting that combination of antioxidants with simvastatin and niacin blunted the favorable effects of lipid-lowering drugs [31]. Acetylcysteine may also be useful as an antioxidant in renal failure patients [32].

There are conflicting data concerning the use of estrogens which are beneficial to endothelium in experimental studies but did not meet expectancies in clinical trials. Supplementation of estrogens to post-menopausal women resulted in even higher coronary events rate. There are inconclusive data concerning the use of folic acid to diminish plasma concentration of homocysteine in uremic patients. In contrast to studies describing hyperhomocysteinemia as a risk factor for cardiovascular events, a recent study found a reverse relationship between homocysteine concentration and both mortality and cardiovascular events in uremic patients [33]. Administration of high-dose folic acid did not affect either mortality or cardiovascular morbidity in that population. In another prospective study a low, rather than a high, plasma homocysteine was an indicator of poor outcome in HD patients [34]. The result may be explained by nutritional feature of homocysteine which is low in malnourished patients.

Statins, among other pleiotropic actions may exert a beneficial effect on the endothelium and increase fibrinolysis. A lag time in a clearly superior effect of statin as compared to placebo in 4S Study [35] and Heart Protection Study [36] suggests an indirect effect of statins, possibly via a beneficial effect on the endothelium. However no lag time was found in recent controlled trials labeled with acronyms REVERSAL [37] and PROVE-IT [38], comparing head-to-head atorvastatin and pravastatin effect on reversing atherosclerotic coronary disease. Both the statins lowered serum C-reactive protein levels – in PROVE-IT Study by over 80%. This suggests a very potent antiinflammatory effect, particularly evident for atorvastatin. Apart from many trials statins have been under-used in uremics, probably due to the high cost of such therapy. As atorvastatin does not need dose adjustment in renal failure (similarly to fluvastatin and pravastatin) it seems to be a drug of choice in patients with chronic kidney disease. It cannot be, however, excluded that new statins like rosuvastatin will be superior to atorvastatin in these patients.

Possible pharmacological prophylaxis of AV graft thrombosis includes antiplatelet agents, anticoagulants, and antiproliferative agents administered either systemically or locally [39]. Recent multicenter placebo-controlled trial of aspirin plus clopidogrel for prevention of AV graft thrombosis was discontinued due to increased risk of bleeding [40].

Clear benefits were established in treatment of acute coronary syndromes with aspirin and heparin, despite the risk of bleeding in chronic renal failure. Also thrombolytic agents or glycoprotein IIb/IIIa inhibitors may be additionally administered if otherwise indicated [41]. Dose adjustments for GFR is necessary for some glycoprotein IIb/IIIa inhibitors. Abciximab appears to be the drug of choice in uremic patients as its dose does not need to be adjusted.

Thrombosis is a serious complication of renal transplantation. No association of renal vein thrombosis with cyclosporine or azathioprine use was demonstrated in a randomized trial [42]. However, cyclosporine A may induce endothelial damage and increase a tendency to thrombosis. In case of hemolytic uremic syndrome/thrombotic thrombocytopenic purpura in a patient treated with calcineurine inhibitor, the drug should be switched or totally withdrawn.

Antithrombotic agents such as heparin and oral anticoagulants are commonly used in uremic patients, although both the treatments need scrupulous monitoring, the same concerns antiplatelet aspirin. Heparin has been extensively used as an anticoagulant during HD procedures. It exerts pleiotropic effects which may be of clinical value. Heparin can release from the endothelium several substances including growth factors (i.e. activin A and follistatin) to the circulation. Recently it was shown that activin A is a potent activator of renal interstitial fibroblasts [43]. The role of follistatin – a natural inhibitor of activin A, may be thus of antifibrotic value. Hepatocyte growth factor, which is also released from the endothelium by heparin, ameliorates renal fibrosis by enhancing extracellular matrix catabolism via both the metalloproteinase and plasminogen activators/plasmin proteolytic pathways [44]. Oral anticoagu-

lants are usually used in uremic patients with persistent atrial fibrillation as a protection from the thromboemboli formation or as prophylaxis of recurrence of deep venous thrombosis. They did not, however, prove to be useful in protection against native AV fistula or synthetic graft thrombosis.

In conclusion, endothelial dysfunction is a prominent feature in patients with renal failure, underlying accelerated atherosclerosis and a high incidence of thromboembolic cardiovascular complications. Specific therapeutic interventions aimed at the endothelium hold exceptional promise for better survival in this population.

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Antioxidants in the treatment of patients with renal failure

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Abstract

Renal failure is accompanied by oxidative stress, which is caused by enhanced production of reactive oxygen species and impaired antioxidant defense. The suggested therapeutical interventions aimed at reducing oxidative stress in chronic renal failure patients are as follows: 1) the use of biocompatible membranes, ultrapure dialysate, and removal of endogenous foci of infection; 2) haemolipodialysis, and electrolysed reduced water for dialysate preparation; 3) administration of antioxidants (α-tocopherol, ascorbic acid, N-acetylcysteine, reduced glutathione); 4) substances possibly affecting oxidative stress indirectly (erythropoietin, sodium selenite). As currently available data have, as yet, provided rather limited evidence for the clinical benefit of antioxidant interventions, at present it is untimely to give practical recommendations with regard to antioxidant treatment of patients with renal failure.

Key words: renal failure, oxidative stress, antioxidant treatment.

Introduction

Renal failure is accompanied by oxidative stress [1,2], which consists in the damage of biological structures by reactive oxygen species due to their excessive generation and impaired efficiency of antioxidant defense mechanisms.

In renal failure patients enhanced reactive oxygen species production is underlain mainly by inflammation [3,4], malnutrition [3], presence of endogenous stable oxidants in the uraemic plasma [5]. In haemodialysis patients the additional stimulus for increased free oxygen radical production can be the haemodialysis procedure itself [6,7]. It is mainly due to inflammatory cell activation caused by insufficiently biocompatible membranes, which is amplified by various bacterial products passing across from the dialysate to the blood compartment [8,9]. Advanced age and diabetes are further factors increasing pro-oxidant activity in renal failure patients [4]. At the same time, impaired activities of endogenous enzymatic free radical scavengers (superoxide dismutase, glutathione peroxidase, catalase) and deficiency of non-enzymatic antioxidants (reduced glutathione, α-tocopherol, ascorbic acid, transferrin, albumin, 17β-oestradiol) aggravate the oxidative stress [1]. Moreover, antioxidant defenses insufficient to shut down the oxidative stress can lead to a chronic and vicious cycle of free radicals causing production of inflammatory mediators that in turn amplify the generation of reactive oxygen species. Either chronic or acute production of free radicals leads to the oxidative modification of lipids, arachidonic acid derivatives, carbohydrates, amino acids, proteins, and deoxyribonucleic acid [1]. In addition, it activates cellular signaling events regulating cell division [10], differentiation [11], and apoptosis [12].

There is growing evidence from experimental and clinical studies that in chronic renal failure oxidative stress can be considered as a potentially important source of patient morbidity and mortality. It may be implicated in the pathogenesis of atherosclerosis [2,13,14], malnutrition [2,13,14], anaemia [15], dialysis-induced amyloidosis [16], and possibly increased risk of cancerogenesis [17] in these patients. Therefore, for some time past various therapeutic interventions have been attempted in order to reduce oxidative stress in chronic renal failure in the hope to improve patient outcome. Therapeutic approaches to reduce oxidative stress in chronic renal failure patients

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Table 1. **Directions of therapeutic interventions considered for reducing oxidative stress in chronic renal failure patients**

A. Reduction of inflammatory cell activation:
1) biocompatible dialysis membranes;
2) ultrapure dialysate;
3) removal of focal infections.

B. Removal of inflammatory mediators:
1) haemolipodialysis;
2) electrolysed reduced water for dialysate preparation.

C. Administration of antioxidants:
1) α-tocopherol;
2) ascorbic acid;
3) N-acetylcysteine;
4) reduced glutathione.

D. Substances possibly affecting oxidative stress indirectly:
1) erythropoietin (?);
2) sodium selenite (?).

are focused on the reduction of inflammatory cell activation, removal of inflammatory mediators and the use of antioxidants [8,9]. Measures considered to date to accomplish this aim are presented in *Tab. 1*.

Reduction of inflammatory cell activation

Reduction of inflammatory cell activation can be attained by the use of biocompatible dialysis membranes, ultrapure dialysate, and removal of focal infections.

Biocompatible dialysis membranes. Haemodialysis procedure performed with a biocompatible (e.g. polyacrylonitrile or polysulfone) dialysis membranes is known to reduce significantly intradialytic oxidative stress [7]. Therefore, one can expect that decreased production of active oxygen species would prevent overproduction of oxidized LDL and enhanced endothelial dysfunction, and, consequently, the risk of atherosclerosis with subsequent cardiovascular complications would be reduced. However, this has not, as yet, been confirmed conclusively, and convincing proofs for cause-effect relationship between membrane biocompatibility per se and accelerated atherosclerosis are still lacking.

Ultrapure dialysate. Reactive oxygen species were found to induce oxidative modification of β_2-microglobulin amyloidosis, and thus, to favour the development of dialysis-induced amyloidosis [16]. Long-term observations disclosed that regular haemodialysis treatment with ultrapure dialysate was associated with a significant decrease in amyloidosis-induced carpal tunnel syndrome compared with pure dialysate [18]. However, it is not, as yet, known whether this phenomenon was affected by the reduction of intradialytic reactive oxygen species production with ultrapure dialysate only or by other factors.

Removal of focal infections. As infections induce oxidative burst of inflammatory cells, removal of focal (i.e. dental, tonsillar, and other) infections, and preventive measures against vascular access infections are of importance.

Removal of inflammatory mediators

Among suggested measures aimed at removal of inflammatory mediators there are haemolipodialysis, and electrolysed reduced water.

Haemolipodialysis relies on the addition of liposomes to the dialysate during the standard haemodialysis procedure [8,9]. The liposomes of 250-300 nm in diameter are comprised of lyophilized soybean phosphatidylcholine bilayer with incorporated α-tocopherol, which form a unilamellar bilayer upon addition to dialysate [8,9]. At the same time, water-soluble ascorbic acid is added directly to the dialysate. These two antioxidants are used together to increase removal of hydrophobic toxins and inflammatory mediators on the one side, and to synergistically scavenge oxidants on the other side, thereby supporting the host's antioxidant defense system. Maintenance of the appropriate ratio between α-tocopherol and ascorbic acid is of crucial importance to avoid their pro-oxidant action. The preliminary experience with haemolipodialysis for the prevention of intradialytic oxidative stress is, however limited, though promising [9].

Electrolysed reduced water. During electrolysis of raw water the active atomic hydrogen with higher reducing activity is released on the cathode. Administration of the dialysate prepared from this electrolysed reduced water during haemodialysis efficiently scavenged hydrogen peroxide and hypochlorite, and ameliorated antioxidant status during one-month treatment [19].

Antioxidants

Among antioxidants α-tocopherol, ascorbic acid, N-acetylcysteine, and reduced glutathione were tried to modify oxidative stress in renal failure.

A-tocopherol. To date, α-tocopherol (vitamin E) was the most frequently used antioxidant to achieve adequate control of oxidative stress in chronic renal failure patients. Short- or long-term administration of vitamin E orally or intramuscularly has been reported to modify beneficially their oxidative status [20-26]. In addition, haemodialysis procedures performed with vitamin E bonded membrane not only ameliorated antioxidative defense [27-33], but also significantly reduced the percentage increase of the aortic calcification index [30], improved carotid intima-media thickness, viscosity and dysmorphism of the red blood cells [30], and haemolysis [32] with resultant reduction of erythropoietin requirements for the treatment of uraemic anaemia [29]. Haemodialysis by vitamin E-coated membrane also prevented dialysis-induced endothelial dysfunction [33].

Although observational studies suggested possible beneficial effect of vitamin E on cardiovascular complications, except for the CHAOS study [34] the vast majority of large placebo-controlled studies on non-renal population (GISSI [35], HOPE [36], SECURE [37], HPS [38]) were rather discouraging,

because they failed to demonstrate a positive effect of vitamin E on cardiovascular event rates.

Meanwhile, the first double-blind placebo-controlled randomized SPACE study [39] performed on 196 haemodialysed patients disclosed a significant decrease in combined cardiovascular event rates in the group of orally treated with vitamin E 800 IU/day over 2 years, but no significant differences in overall mortality and mortality from cardiovascular disease were observed. However, in view of negative influence of vitamin E on the blood serum level of protective HDL_2 cholesterol, its safety in the long-term antioxidative treatment needs to be determined [2].

Ascorbic acid represents one of the most prominent antioxidants, exerting beneficial effects by an inhibition of lipid peroxidation and by reducing endothelial dysfunction [40]. Although in chronic renal failure patients deficiency of vitamin C can be observed, its administration in these patients requires deliberation. Vitamin C in food or as supplementation may lead to its excessive serum levels, resulting in hyperoxalaemia that may contribute to vascular disease in uraemic patients [41]. In addition, in the presence of transition metals like iron, ascorbate may give rise to an increased generation of antioxidants, and ascorbylation may impose additional carbonyl stress to uraemic patients, particularly in the presence of high blood glucose levels [40]. Therefore, 60 mg of oral vitamin C are currently recommended for chronic kidney patients [42], while in case of suspected subclinical ascorbate deficiency 1-1.5 g of oral vitamin C per week or 300 mg parenteral ascorbate per dialysis session are recommended, respectively [43].

N-acetylcysteine. In the recently published randomized controlled trial [44], treatment with the reduced thiol-containing antioxidant N-acetylcysteine (600 mg orally twice a day for a median of 14.5 months) significantly reduced cardiovascular events by 40% in the treated group compared with the placebo group. However, no effect was reported on total or cardiovascular mortality.

Reduced glutathione. Intravenous administration of exogenous reduced glutathione (tationil) alone [45] or in combination with the use of vitamin E bonded dialysis membrane [46] significantly improved uraemic anaemia. Some investigators [46] believe that combined use of the vitamin E bonded membrane and intravenous reduced glutathione seems to be the best antioxidant therapy so far, with significant saving of recombinant human erythropoietin dose.

Substances with possible indirect antioxidant action

Also some substances with possible indirect antioxidant action, e.g. erythropoietin, and sodium selenite, seem to be helpful for antioxidant treatment in renal failure.

Though erythropoietin is not an antioxidant, it is suggested that in chronic renal failure it can reduce oxidative stress indirectly by the correction of uraemic anaemia, and consequently, the rise in glutathione content in the blood. However, up-to-date reported studies did not provide univocal results. During recombinant human erythropoietin therapy some authors did not found significant changes in the oxidative stress intensity [47] or antioxidant status [48], while others observed positive effects [49-51].

Selenium deficiency, frequently observed in chronic renal failure patients [48], may contribute to the impairment of activity of glutathione peroxidase, an enzymatic antioxidant belonging to selenoproteins. Correction of selenium deficiency by sodium selenite given intravenously [52] significantly increased red blood cell glutathione peroxidase activity, while selene-rich yeasts given orally did not affect this enzyme activity [53]. Influence of selenite supplementation on other components of the antioxidant system remains unclear.

Conclusions

There are many prerequisites suggesting possible beneficial effects of therapeutic interventions aimed at reducing oxidative stress in chronic renal failure, and the recently published results of two randomised placebo-controlled clinical trials [39,44] are particularly promising in this respect. However, according to Steinberg and Witztum [54] several crucial questions still remain unanswered: 1) have the clinical trials been done with the right antioxidants at the right doses? 2) was the effectiveness of these antioxidants investigated properly? 3) are the right markers for oxidative stress and for the effectiveness of the antioxidants identified? 4) were the patients for the antioxidative therapies chosen accurately? 5) have the trials been started early enough, and have they lasted long enough? 6) are the species differences such that the results in animal models do not extrapolate to humans? Answering these questions will enable to design suitable antioxidant protocols and to evaluate their effectiveness in chronic renal failure. As currently available data have, as yet, provided rather limited evidence for clinical benefit of antioxidant interventions, at present it is untimely to give practical recommendations with regard to antioxidant treatment of patients with renal failure.

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Heart failure in the patients with chronic kidney disease

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Abstract

Heart failure is highly prevalent in the population with chronic kidney disease. Upon starting dialysis, 37% of patients will have had a previous episode of heart failure, doubling the risk of death. Both systolic and/or diastolic function may be impaired. 15% of patients starting dialysis therapy have systolic dysfunction of the left ventricle. The prevalence of diastolic dysfunction at dialysis inception is unknown, but is likely to be high. Either systolic or diastolic dysfunction can lead to clinically evident congestive heart failure. Hypertension and coronary heart disease are important causes of myocardial dysfunction in end-stage renal disease. Individuals with chronic kidney disease are at a very high risk for the development and progression of cardiovascular disease. The increased risk of cardiovascular disease is due to a higher prevalence of both traditional risk factors as well as nontraditional "uremia-related" risk factors. The prevalence of coronary artery disease (CAD) approaches 40% among patients starting dialysis. About 70 - 80% of these patients have hypertension. Anaemia is a known risk factor for left ventricular hypertrophy (LVH) and dilatation, heart failure and death. The diagnosis and treatment of heart failure in the patients with chronic kidney disease (CKD) are similar to that reccomended for patients without CKD. The potent drugs like ACE-I, AT-1 antagonists, β-receptor antagonists are the main tools in nowadays treatment of CHF. New therapeutic regiments using natriuretic peptides are being evaluated in clinical settings.

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Introduction

The cardiovascular system is closely related to function of the kidneys. Renal insufficiency can affect cardiac performance leading to its failure which consequently worsens renal function. The fact, that impairment of one component of the cardio-renal system aggravates dysfunction of the other is clinically very important.

Heart failure (HF) is a specific term used to define the clinical syndrome when the heart is unable to pump enough blood to supply the metabolic needs of the body [1]. Subjects with myocardial failure can have symptomatic HF or asymptomatic ventricular dysfunction. Symptoms of exercise intolerance are typically assessed by the New York Heart Association (NYHA) functional classification.

About half of all deaths in end-stage renal disease (ESRD) patients are attributable to cardiac causes [2]. Heart failure and coronary heart disease (CHD) are highly prevalent in this population.

Epidemiology

Upon starting dialysis, 37% of patients will have had a previous episode of heart failure, doubling the risk of death [3]. The remaining patients will develop heart failure at a rate of about 10%/year [4]. Both systolic and/or diastolic function may be impaired. 15% of patients starting dialysis therapy have systolic dysfunction of the left ventricle [5]. The prevalence of diastolic dysfunction at dialysis inception is unknown, but is likely to be high [6]. Either systolic or diastolic dysfunction can lead to clinically evident congestive heart failure (CHF). Risk factors for new onset CHF include hypertension, older age, anaemia and coronary heart disease [7]. Hypertension and CHD are important causes of myocardial dysfunction in ESRD.

Coronary heart disease in patients with chronic kidney disease

Individuals with chronic kidney disease are at a high risk for the development and progression of cardiovascular disease [8]. The prevalence of CAD approaches 40% among patients starting dialysis. 22% of them have stable angina, while 18% will have suffered from a prior myocardial infarction. The symptoms of myocardial ischaemia in dialysis patients are the same as those observed in other groups, although silent ischaemia may be more common because of the higher prevalence of diabetics [2]. The increased risk of cardiovascular disease is due to a higher rate of both traditional as well as nontraditional "uremia-related" risk factors [9].

Most important traditional risk factors are: older age, male gender, hypertension, lipid abnormalities (increased level of total and LDL cholesterol, decreased level of HDL cholesterol), diabetes, smoking, physical inactivity, left ventricular hypertrophy, family history of cardiovascular disease.

For the nontraditional risk factors we account: albuminuria, anaemia, abnormal calcium/phosphate metabolism, hyperhomocysteinemia, extracellular fluid volume overload, oxidative stress, inflammation, malnutrition, thrombogenic factors.

CHD is usually the result of critical coronary artery disease, but in 27% of hemodialysis patients ischaemic symptoms are caused by other changes, like: small vessel disease (caused by hypertension, diabetes mellitus and calcium-phosphate deposition), reduced capillary density and abnormal myocyte bioenergetics [10]. LV hypertrophy predisposes to ischaemic symptoms by reducing coronary reserve.

The patology of CHD in patients with renal insufficiency is complex. It's important to note that, in uremic patients the atherosclerotic plaque is more calcified [11]. Morever not only epicardial arteries, but also small arterioles are involved in atherosclerosis. The left ventricular hypertrophy often coexists. In the beginning it is concentric, followed by excentric hypertrophy which leads to the dilatation of the left ventricle.

The coronary flow reserve is decreased [12]. Increased concentration of NO inhibitors due to decreased accessibility of nitric oxide is present [13]. Hypertrophy and hyperplasia of the coronary vessels further limit its dilatation ability [14]. Decreased amount of capillary vessels in myocardium impairs oxygen diffusion.

The following risk factors lead to cardiomyopathy in patients with chronic kidney disease:

- LV volume overload: salt and water overload, arteriovenous fistula, anaemia
- LV pressure overload: hypertension, aortic stenosis, arteriosclerosis.

Other causes are: hypoalbuminemia, small and large coronary vessel disease [15].

Hypertension in patients with chronic kidney disease (CKD)

About 70-80% of these patients have hypertension. The prevalence of high blood pressure increases as GFR declines [16]. Three out of four of patients starting dialysis have left ventricular hypertrophy (LVH) [17]. For dialysis patients, each 10 mmHg increment in blood pressure is associated with a 48% higher risk of LVH [18]. Risk factors for LVH apart from systolic blood pressure, include: anaemia, age and gender. Reducing blood pressure slows the rate of loss of renal function in CKD [6]. On the other hand NHANES III data suggest that in the majority of patients with renal insufficiency hypertension is poorly controlled. 2/3 of them had blood pressure > 140/90 mmHg. Only 11% individuals had adequate values.

Angiotensin converting enzyme inhibitors and angiotensin receptor blockers should be considered the preferred antihypertensive agents. Calcium channel blockers are recommended to be used only as a part of a multidrug regimen in combination with ACE inhibitors and angiotensin receptor blockers [8]. Usually multidrug therapy is needed. According to the standards, the uremic patients require more intensive treatment of high blood pressure (INC VI):

- individuals without proteinuria – less than 130/85 mmHg
- individuals with proteinuria (> 1 g/day) and/or diabetes it should be lower – 125/75 mmHg.

The optimal pressure values for dialysis patients is not clear, as low blood pressure has been associated with increased mortality in this group [19].

It is worth to note that, in the Hypertension Optimal Treatment (HOT) trial participants with elevated baseline serum creatinine were two- to threefold more likely to experience a major or fatal CVD events compared to subjects with normal serum creatinine levels [20]. The prognostic significance of serum creatinine levels was also reported in the Heart Outcomes Evaluation Protection Study (HOPE) [21].

Dyslipidemia in patients with chronic kidney disease (CKD)

The most important abnormalities of uremic dyslipidemia are: elevated levels of trigliceryde trigliceryde-rich particles (VLDL and IDL) and LDL-cholesterol, increased Lp(a) lipoprotein level, low concentration of HDL-cholesterol. Total cholesterol level can be normal, although this is not the rule [22]. The prevalence of dyslipidemia in CKD is associated with the level of GFR and the level of proteinuria [23].

The patients with CKD and hyperlipidemia should be treated according to the National Cholesterol Education Program (NCEP) guidelines [2]. The target LDL cholesterol level is ≤100 mg/dl. The HMG-CoA reductase inhibitors (statins) are the most effective drugs. Fibrates are also effective, but they are excreted by the kidney, that is why dosage reduction is required.

Anaemia in patients with CKD

Anaemia is a special risk factor in patients with CKD. This fact well recognized by nephrologists is often unrealised by other specialists, also cardiologists. It influences left ventricular hypertrophy (LVH) and dilatation, heart failure and death [24].

Anaemia with haemoglobin levels < 12 g/dl is present in more than half of patients with advanced heart failure. Its incidence increases with higher NYHA classes. Several observational studies have suggested that anaemia (haemoglobin levels 6 - 12 g/dl) is an independent predictor of mortality in dialysis patients [6]. It has been shown that haemoglobin normalization may prevent progressive LV dilatation in patients with normal cardiac volumes at baseline [25]. Results of recent studies indicated that partial or complete correction of anaemia with erythropoietin decreases the cardiac output and heart rate, and induces a partial regression of the LVH, principally in relation with decreased left ventricular end-diastolic diameter [26]. Foley et al. postulated, that normalization of haemoglobin in haemodialysis patients with asymptomatic cardiomyopathy prevented the development of further left ventricle dilation [25]. The Canadian Normalization of Hemoglobin study (haemodialysis patients with either LVH or LV dilatation randomly treated to hemoglobin levels of 10 or 13.5 g/dl) showed that normalization of haemoglobin led to clinically significant improvements in quality of life, while survival rate were similar in both target groups [6].

Hyperparathyroidism in patients with CKD

High level of parathyroid hormone is an important factor in the genesis of myocardial fibrosis [27]. Hyperparathyroidism is associated with LV hypertrophy. Some studies have shown improvement in left ventricular function and size after parathyroidectomy [6].

Diagnosis of heart failure in patients with CKD

The diagnosis consists of:

- symptoms of heart failure
- declinations in physical examination
- ECG
- chest X-ray
- echocardiogram.

Echocardiography is the most important tool for the diagnosis of CHF in CKD patients. Especially echocardiography in the patients starting dialysis revealed a variety of abnormalities. Most common was concentric LV hypertrophy found in 42% of patients, eccentric LV hypertrophy in 23%, isolated LV dilatation in 4% and systolic dysfunction in 16%. Only 16% had a normal echocardiogram [28]. Similar data (about 75% patients with LVH) showed USRDS (United States Renal Data System) [29].

However, LV volume fluctuates in haemodialysis patients. Therefore it is necessary to perform echocardiography at the patients "dry weight" – the day after dialysis. In patients with anaemia the increase in cardiac preload alters the pattern of Doppler signals that is used for the evaluation of diastolic function.

Treatment of heart failure in patients with CKD [2]

The data are lacking, because patients with significant renal impairment have been exluded from randomized studies. Few little trials indicated that ACE inhibitors and β-receptor antagonists improve outcomes also for patients with heart failure in the CKD and ESRD populations.

ACE Inhibitors are metabolized in kidneys, therefore their dose has to be adjusted according to the renal insufficiency. This, however, is not a problem in the case of the angiotensin II receptor type 1 (AT-1) antagonists, which are metabolized predominantly by liver. Nevertheless some of them, like valsartan, also require dose adjustments in advanced renal failure (GFR < 30 ml/min). A separate problem is the risk of hyperkalaemia and decrease of the glomerular filtration as the consequence of the inhibition of the renin-angiotensin-aldosterone system. According to the guidelines, serum levels of potassium and creatinine should be initially monitored every 4 weeks and at least twice a year on a regular basis. A discontinuation of the drug should be considered when serum potassium exceeds 5.5 mmol/L or creatinine increases more than 30% of the basal (pre-treatment) values [16].

There was one prospective placebo-controlled trial – 114 dialysis patients with dilated cardiomyopathy were randomized to receive either carvedilol or placebo in addition to standard therapy. All patients were followed up for two years. After two years, 51.7% of the patients died in the carvedilol group, compared with 73.2% in the placebo group ($p < 0.01$). These data suggest the use of carvedilol in all dialysis patients with chronic heart failure [30]. In such patients carvedilol has good kinetic characteristics – its hepatic metabolism does not require dose adjustments in case of impaired renal function.

Loop diuretics are complying in patients with symptoms of CHF in renal failure. Their effect will be attenuated in cases with advanced renal insufficiency, but not as severly as thiazide diuretics, which usually become ineffective with a GFR less than 30 ml/min. The effects of aldosterone antagonists are unpredictictable in patients with CKD – they can induce hyperkalemia, in combination with ACE inhibitors and β-receptor antagonists in the setting of reduced GFR.

It has been demonstrated that the use of diuretics alone, by reducing the effective plasma volume, results in further activation of the neurohormonal axis. The administration of diuretics rises activation of the renin-angiotensin-aldosterone system (RAAS). Multiple studies have shown that, when diuretics are used alone, one of their effects on the kidney is to significantly decrease glomerular filtration rate. Acute Decompensated Heart Failure National Registry (ADHERE) database indicates than more than 80% of patients are managed with diuretics. It makes sense when patients are congested. But it must be emphasized that diuretics are really a double-edged sword in heart failure. They activate neurohormones, they cause potassium loss, and they have detrimental effects on renal function.

It is worth to note that, aggressive therapy for advanced heart failure can aggravate renal dysfunction. Estimation creatinine clearance may predict this situation better than baseline creatinine levels.

Increased experience with natriuretic peptides suggests that their administration might facilitate diuresis with less compromise of renal function. Probably they can also limit the dilatation of the heart and maybe activation of pathways at a cellular and molecular level for cardiac remodeling.

Each of modern medications alone causes a significant reduction in mortality; but the cumulative risk reduction when combined therapy (ACE-I, β-receptor antagonists and aldosterone antagonists) is used brings a 66% risk reduction [31].

Digoxin is useful in patients with atrial fibrillation and heart failure, and it improves exercise tolerance in nonuremic patients with symptomatic LV systolic dysfunction. Digoxin should be considered for similar patients with CKD or ESRD.

Conclusions

More than one a third of patients starting dialysis have clinical symptoms of heart failure. On the other hand, congestive heart failure is a common and crucial contributor to the progression of chronic renal disease. If we can help prevent renal dysfunction in heart failure, we are likely to be much more successful in dealing clinically with such patients. Therefore close cooperation between cardiologists and nephrologists is needed. The potent new drugs like ACE-I, β-receptor antagonists and angiotensin receptor blockers are the main tools in nowadays treatment of CHF. New therapeutic regiments using natriuretic peptides are being evaluated in clinical settings.

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What is new in therapy of glomerulonephritis in the 2003/2004

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Abstract

The article reviews reports and opinions dealing with management of human glomerulonephritis that have appeared in the years 2003/2004. The following glomerulopathies have been covered: primary focal and segmental glomerulosclerosis, IgA nephropathy, membranous nephropathy, lupus nephritis, ANCA positive vasculitis and HCV-positive membranoproliferative cryoglobulinemic glomerulopathy. Aside from original studies, expert opinions and recommendations have been cited.

Key words: glomerulonephritis therapy, primary and secondary glomerulopathies.

The problems of pathogenesis and treatment of human glomerular diseases are particularly complex and multifactorial, as much as clinical management of glomerulonephritis. Despite a notable improvement made in the past decade in this field, in particularly regarding secondary glomerular disease, satisfactory results have not been reached in majority of glomerulonephritides. Therefore, this presentation aimed at underlining novel approaches to management of these diseases, is based upon my personal, based selection of, in my opinion, interesting and representative reports, that have been published in the years 2003/2004. Thus it doesn't mean that the reports and opinions cited herein reflect a complete upate of this complicated and multifaceted subject.

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Primary focal and segmental glomerulosclerosis (FSGS)

A prolonged treatment with corticosteroids is a first therapeutic option. In resistant cases cytotoxic agents, cyclosporine, mycophenolate mofetil (MMF), plasmapheresis or LDL-apheresis have been tried with variable results. In adults resistant to corticosteroids several months' course of cytotoxic therapy is warranted. Some benefit has been reported of treatment with MMF, although in uncontrolled studies. Nonetheless, as for now, a 6 months trial of cytotoxic drugs or MMF may be recommended in steroid-resistant FSGS to identify few responsive cases. Plasma- or lipopheresis is also promising but awaits confirmation of effectivity in future trials [1]. Some 50-70% of steroid-resistant FSGS cases will have a marked reduction of proteinuria as result of treatment with cyclosporine A (CsA). Apropriate titration of dosage to avoid nephrotoxicity is necessary and a course of up to 12 months is advisable without a substantial risk of cyclosporine-dependence. Given the above, CsA therapy could be regarded as primary treatment in patients with FSGS at high risk of steroid toxicity [2]. The rationale of employing corticosteroids in therapy of FSGS has been questioned by the group of Ron Falk (Glomerular Disease Collaborative Network; [3]). An aggressive and multifactorial therapy of collapsing glomerulopathy, a variant of FSGS, has been proposed by Gerald Appel and his coworkers [4]. It includes steroids or CsA, ACE-inhibitors, AII receptor blockers and statins. Moreover, 6-12 months' course of AII receptor antagonist losartan (50 mg/day) was effective in ameliorating nephrotic syndrome in normotensive patients with FSGS resistant to immunosuppressive treatment [5]. Contrary to the above cited opinion of R. Falk and coworkers, Indian authors demonstrated

in a controlled trial that patients with FSGS should be given steroids for at least 4 months before they could be regarded non-responsive to these drugs [6]. In a study assessing long-term effects of CsA or chlorambucil treatment (6 months trial) of patients with nephrotic syndrome due to FSGS, investigators of the German Collaborative Glomerulonephritis Study Group [7] revealed that at 4 years after the trial mean serum creatinine in group receiving CsA was appx. 1.7 mg/dl, while in that treated with chlorambucil was 1.9 mg/dl (difference not statistically significant). Total remission of nephrosis was seen in 28% of patients taking CsA. Thus this form of therapy appears to be relatively safe and effective in the FSGS, also in the long run, however the results were not significantly better than those of chlorambucil treatment. Stewart Cameron has also contributed to the above cited discussion on usefulness of corticosteroids in FSGS by stating that nowadays, contrary to the past, a 4-6 months course of steroids is warranted in all adult nephrotic patients with FSGS to establish if they fall into the 20-30% who will thus achieve a remission of proteinuria [8]. These steroid-sensitive patients have a benign prognosis as to progression of renal disease and their management could rely on long-term (24 months) low dose steroids and/or CsA (up to 12 months). A study reported in children, but equally applicable to young adults with steroid-resistant FSGS, proposed combined use of MMF and ACE-inhibitors or AII receptor blockers if all other measures failed [9]. After 6 months of such medication proteinuria was reduced by 72% and this level maintained for at least 24 months. Nonetheless this study, as many other similar reports, has suffered from a low number of cases.

IgA nephropathy

Among many reports on beneficial effect of anti-angiotensin treatment in this nephropathy, a group from Korea [10] has documented an additive antiproteinuric influence of combined ACE-inhibitor and AII receptor blocker. Interestingly, in a group of type 2 diabetic nephropathy patients treated in parallel, such synergism against proteinuria could not be observed. A controlled trial assessing effect of steroid therapy in patients with moderate proteinuria (0.5 - 2.0 g/d) was performed in Japan [11]. The result revealed not only significant reduction in proteinuria in steroid-treated patients, but also remarkable improvement of renal histology assessed on repeated biopsy at 3 years' follow-up. Another controlled trial in Japan featured over 5 years' observation of patients treated with low dose steroids (20 mg tapered to 5 mg/day) for histologically moderate IgA nephropathy. As result, this dosage, while reducing proteinuria, was ineffective in arresting deterioration of glomerular filtration. Authors concluded that higher doses of corticosteroids are mandatory in management of this entity [12]. On the other hand, histopathologically severe crescentic IgA nephropathy was successfully managed with pulse methylprednisolone and intravenous cyclophosphamide repeated monthly for 6 months. Marked histopathologic improvement was noted on repeat biopsy along with significant reduction in proteinuria. At 36 months' follow-up end-stage renal disease was observed in 1/12 patients of treated group versus 5/12 historical controls [13]. A 10 years' follow-up study was performed in Greece on patients with IgA nephropathy and heavy proteinuria (>3 g/d). Results revealed beneficial effect of treatment with corticosteroids and azathioprine (24 months) on progression of glomerular disease to end-stage renal insufficiency [14]. In advanced cases of nephropathy presenting with reduction in glomerular filtration (creatinine > 1.5 mg/dl) it may be advisable to combine pulse methylprednisolone treatment with tonsillectomy. This approach significantly decreases rate of progression to ESRD in over 5 years' follow-up [15]. Similarly advanced (creatinine >2 mg/dl) and progressing (25% increase of serum creatinine in the past 3 months) noncrescentic IgA nephropathy was evaluated for response to cyclophosphamide pulse therapy (750 mg/m^2 monthly for 6 months) and low dose prednisolone by Rasche FM et al. [16]. This treatment reduced the rate of renal function loss from 16% to 4% along with significant decrease of proteinuria and was therefore regarded effective in this subgroup of patients. A group of children or young adults with moderate proteinuria (normocholesterolaemia) and mild histopathological changes responded favourably to combined treatment with fluvastatin and dipyridamole (12 months) in terms of diminution in proteinuria, hematuria and serum creatinine concentration [17]. Finally, a group of 110 patients with mesangial proliferative nephropathy and IgM deposits (IgM nephropathy) was observed for 15 years by Finish investigators: 29% were resistant to corticosteroids and 80% of steroid-sensitive were steroid-dependent [18].

Membranous nephropathy

Despite extensive clinical investigations in the past 2 decades, the jury is still out on optimal scheme of management of this type of glomerulonephritis. In particular, the efficacy and safety of majority of protocols employing corticosteroids and alkylating agents must be regarded at least controversial. In a recent review, a group of Chapel Hill, USA, suggests more selective, innovative strategies aimed at modulating immune response to pathogenic antigen, inhibition of B and T cell activation, blockade of complement cascade, interference with peroxidation of GBM components and others [19]. With regard to steroid and cytotoxic drugs, the authors of original scheme of treating membranous nephropathy with alternating monthly schedule admit that although significant antiproteinuric effect was noted, long-term nephroprotective influence was uncertain and severe side effects were observed in patients with impaired renal function [20]. CsA has been shown in a number of controlled trials to markedly diminish proteinuria and arrest progression of renal failure in at least 2/3 of patients characterised as being at high risk of progression (reviewed in [21]). The evidence in favour of MMF efficacy in these patients is much weaker and derived from pilot studies: half of the patients will have a 50% reduction in proteinuria and no effect on renal function. Down the line suggested by the above cited selective approach to treatment, the group of G. Remuzzi reported on a successful use of anti-B cell (CD20) monoclonal antibody, rituximab in patients with membranous nephropathy at high risk of progression to ESRD [22]. Specifically after 12 monthly infusions renal function sta-

bilized and proteinuria decreased to <0.5 g/d. Finally, a report from Hong Kong described effective treatment with tacrolimus of 3 patients with membranous nephropathy nonresponsive to steroids and cytostatics: there was complete remission in 1 and partial in 2 patients [23].

Lupus nephritis

It is now recognized that this disease represents rather a clinical syndrome, than the unique pathogenic and clinical entity, therefore there cannot be a uniform therapeutic approach to all the types and patient subgroups. Each treatment strategy includes induction and maintenance phase and recommendations for both phases are still controversial. Whereas induction therapies outlined by the NIH (intravenous cyclophosphamide pulse therapy for diffuse proliferative lupus nephritis) and the Mayo Clinic trials are more or less widely accepted, the maintenance therapy abounds controversies as to whether cyclophosphamide or azathioprine are effective and safe in this regard [24]. Recently, Contreras G et al. [25] published results of their prospective controlled trial comparing efficacy and safety of 3 regimens aimed at maintaining results of remission induction: quarterly pulse cyclophosphamide, oral azathioprine and oral MMF. Following the pulse-cyclophosphamide induction phase that caused remission in 83% patients, they obtained much better results in maintenance phase (72 months event-free survival and relapse-free survival, as well as incidence of hospitalization and side-effects) in the azathioprine and MMF groups than in the cyclophosphamide group. New drugs currently employed in other autoimmune diseases haven't been extensively used in treatment of lupus nephritis: methotrexate, CsA, high dose intravenous immunoglobulins [26]. The same applies to immunosuppressive drugs used in transplantation (MMF, tacrolimus). There are interesting attempts with monoclonal antibodies against immune cells, cytokines and components of the complement system, as well as with autologous bone marrow transplantation in treatment of severe lupus nephritis. Recently, a group from the Heidelberg University compared outcomes of lupus nephritis in patients treated at this institution in the years 1980-1989 and 1990 through 2000. In the later decade there were lower rates of renal failure and fewer histological signs of chronicity at diagnosis. Thus, although the treatment schedules were not significantly different, the outcome of disease was clearly better in the recent decade (40% of ESRD in the years 1980-89; no ESRD in the 1990-2000, [27]). A new immunosuppressive drug developped in Japan, mizoribine has been used to relieve flare of lupus nephritis in a pilot prospective study of 6 patients [28]. Oral pulse therapy resulted in reduction of proteinuria, serum anti-dsDNA antibody titers as well as significant histological improvement in 1 patient who has been rebiopsied after treatment. Yet another novel drug, rituximab (anti-CD20 monoclonal antibody) has been successfully employed (complete remission achieved) by a group from Navarra (Italy) in management of lupus nephritis refractory to cytostatics and steroids [29]. Evaluation of safety and efficacy of another monoclonal antibody, anti-CD 40L was performed by investigators from NIH, Bethesda, in 28 persons with proliferative lupus nephritis. The study was terminated prematurely because of thromboembolic phenomena occurring in some patients, however reduction in proteinuria and anti-dsDNA titers was observed in few persons available for assessment of efficacy [30]. Also from the NIH originated a review article summarizing current approach to treat lupus membranous nephropathy [31]. It was concluded that the patients with this entity should be treated from the earliest stages of the disease with angiotensin antagonists to minimize proteinuria, whereas after development of nephrotic syndrome active immunosuppression should be employed including corticosteroids, cyclosporine, MMF and cyclophosphamide.

ANCA positive renal vasculitis

The second wave clinical trials launched by the European Vasculitis Study Group (EUVAS) was aimed at testing new therapeutic approaches. Results of the part of the CYCLOPS (pulsed vs. oral continuous cyclophosphamide in generalized vasculitis) trial from the First Medical Department, Charles University (Prague) were recently published [32]. It appears from the outcome that mortality was higher in the oral cyclophosphamide arm, so was the cumulative dose of the drug and infection rate, although while remission rate was lower, the relapses were less frequent in the oral cyclophosphamide group. New trials by EUVAS include comparison of MMF and azathioprine in maintenance of remission (IMPROVE). The efficacy of both anti-TNF antibody and soluble TNF receptor in management of ANCA (+) vasculitis have been probed recently. A study from Bologna revealed that plasma exchange treatment (PE) improves prognosis in acute phase of ANCA (+) crescentic glomerulonephritis: in patients receiving both PE and steroids plus oral cyclophosphamide there was less mortality and lower rate of transfer to end-stage renal disease than in those given immunosuppressive drugs alone [33]. Tetracycline derivatives inhibit production and activity of matrix metalloproteinases; a single case report demonstrated remarkable subsidance of proteinuria in crescentic glomerulonephritis with tetracycline [34].

Miscelanous

Rather futuristic approach of treating glomerular diseases has been reviewed by Schena FP and Abbattista MR [35]: infusion of bone marrow-derived stem cells could repair injured glomeruli. Data from experimental animal studies indicate that this strategy may be employed in treatment of human glomerulopathies in the near future. The issue of combined ACE and AII receptor blockade in reducing proteinuria in glomerulonephritis has been tackled by few studies. The G. Remuzzi's group achieved a synergistic antiproteinuric effect when both drugs were used in concert [36], while investigators from Bern (Switzerland) observed reduction in serum matrix metalloproteinase activity with ACE inhibitor, but not with AII receptor blockers, or combined use of both anti-angiotensin drugs [37]. The major advantage of LDL-apheresis and

immunoadsorption as selective procedures over nonselective plasmapheresis in management of certain glomerulopathies has been underlined by Sułowicz W and Stompór T [38].

In patients with HCV infection and cryoglobulinemic glomerulonephritis (membranoproliferative, MPGN), a 12 months course of interferon alpha (IFNα) and ribavirin results in sustained virologic response, along with improvement of clinical and histologic features of glomerulopathy [39]. The treatment with IFNα alone may be complicated by relapses of HCV viremia and renal disease after discontinuation of therapy. In cases of rapidly progressive MPGN the treatment is initiated by pulses of methylprednisolone plus oral prednisolne with cytostatic, followed by the above antiviral therapy. In patients with genotype Ib HCV cryofiltation (double filtration plasmapheresis with a cooling unit) combined with IFNα and ribavirin is recommended [40].

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What is new in peritoneal dialysis in the years 2003-2004

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Abstract

This review paper describes new data on the role of aquaporins in peritoneal function, peritoneal membrane permeability, peritoneal dialysis (PD) adequacy and peritoneal membrane histology, new aspects of the use of PD solutions alternative to glucose ones and indications for use of peritoneal catheters with different configurations.

Results of main studies, published predominantly in 2003-2004, are presented and discussed. Clinically important news, although preliminary in some cases, are also included.

Key words: aquaporins, peritoneal membrane, dialysis solutions, catheters.

Introduction

The following subjects will be discussed:

1. The role of aquaporins (APQs) in peritoneal function,
2. Peritoneal membrane permeability, peritoneal dialysis (PD) adequacy and peritoneal membrane histology,
3. New aspects of the use of PD solutions alternative to glucose ones,
4. Indications for use of peritoneal catheters with different configurations.

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The role of AQPs in peritoneal function

When expression of cellular water channels – AQPs – was found in kidney tissue, exploration of peritoneum for AQPs was also initiated. In human peritoneum mRNA for AQP1, AQP3 and AQP4 was shown [1]. The localization of AQP1 protein in peritoneal mesothelial cells was confirmed by double immunohistochemical staining of the mesothelial lining of human peritoneal membrane. Immunohistologic studies revealed different localization of AQP1 and AQP3 in human peritoneal mesothelial cells, with apical localization of AQP1 and basolateral localization of AQP3 [2]. In mice, the presence of mRNA for AQP1, AQP3, AQP4, AQP7 and AQP8 was reported in peritoneum [3]. In the rat peritoneum, mRNA for AQP1 and AQP4 was shown [4].

AQPs, which are present in human peritoneum, were also found in other human organs. AQP1 was detected in kidneys (proximal tubule, descending thin limb of the loop of Henle, the descending vasa recta), chorid plexus, eye, gall bladder, lung, red blood cell, endothelium of non-fenestrated capillaries. AQP3 is present in kidney collecting ducts (basolateral membrane), lung, gastrointestinal tract, chorid plexus and eye. AQP4 was found in brain, lung and kidney [2,5].

Important progression of investigations in cell cultures was achieved with detection that glucose itself or hyperosmotic conditions, caused by high glucose concentrations (4%), increase expression of mRNA and synthesis of AQP1 protein in endothelial cells of human umbiliculus vein [6] and in rat peritoneal mesothelial cells [7]. These data suggest that during PD a number of AQP1 may increase due to action of hyperosmotic solutions containing glucose, if cells responsible for AQPs formation are capable to answer on stimulus like hyperosmotic glucose solution. Chronic exposition of peritoneum on dialysis solution with low pH (3.5) causes a decrease of mRNA for AQP1 and AQP4 in the rat model of PD [4].

Not all AQPs are involved in transperitoneal ultrafiltration. Studies on AQP1-null mice or AQP4-null mice have shown that AQP4 plays no significant role in peritoneal water permeability,

whereas in AQP1-null mice the rate of increase in the volume of a hypertonic dialysate was reduced to about 40% of the level seen in control mice [3]. Results of these studies also indicate that a presence of AQP1 is not the only condition causing transperitoneal ultrafiltration under hypertonic circumstances. Investigations on rats with renal-vascular hypertension revealed that greater ultrafiltration is accompanied by higher expression of AQP1 and AQP4 [8]. Expression of AQPs may be decreased by administration of angiotensin converting enzyme inhibitors (ACEI) or drugs which are antagonists for angiotensin II receptor (AIIA). Action of ACEI or AIIA results in decreased transperitoneal ultrafiltration [8].

Reduced expression of AQPs as a cause of deteriorated ultrafiltration should be suspected when patients with decreased ultrafiltration demonstrate impaired sodium sieving, but peritoneal transport of small solutes is not increased [2].

Attempts to demonstrate a decreased number of AQP1 in patients with impaired peritoneal ultrafiltration capacity were, however, not successful. Changes in AQP1 expression were not shown during peritonitis in human [9] and in the rat model of peritonitis. A presence of AQP1 was also revealed in a long-term patient with impaired transcellular water transport [10]. A question arises, what is really important – a number or a function of AQPs. It cannot be excluded that glycation of proteins of AQPs is a reason for loss of their physiological functions [2].

Peritoneal membrane permeability, PD adequacy and peritoneal membrane histology

Long-term exposure of peritoneal membrane to bioincompatible dialysis solutions and episodes of dialysis-related peritonitis lead to functional and structural changes in peritoneum of PD patients.

Functional changes of peritoneal membrane are relatively easy to check with repeated performance of peritoneal tests, like peritoneal equilibration test – PET [11], standard permeability analysis – SPA [12], personal dialysis capacity – PDC [13,14]. In 2003, reference values of SPA were established [15], and PET was modified by the use of a radiopharmaceutical: ^{99}Tc – diethylenetriaminepentaacetate – ^{99}Tc – DTPA, which was intravenously injected at the end of peritoneal instillation of 2 L of 2.5% glucose – containing PD solution as a bolus [16]. Comparing to standard PET, nuclear PET may have the added advantages of simplicity and a possibility of measuring total clearance (PD + renal) in the same sitting. All afore-mentioned tests determine transperitoneal movement of small solutes. SPA and PDC additionally determine peritoneal ultrafiltration capacity, function of water channels and peritoneal transport of large molecules. However, standard PET remains the most popular method of functional evaluation of peritoneal membrane.

Numerous papers indicate changes in peritoneal permeability in the course of PD treatment. An increase in peritoneal permeability is predominantly described. It occurs usually after at least 2 years of PD duration [17-21]. Increments in peritoneal permeability were also observed in the rat model of PD [22]. Reports on decreasing permeability of peritoneum during PD treatment also appear in the scientific literature [19,23-27].

Recent studies indicate that the normal anatomic peritoneum (the mesothelium and associated surface coatings, stagnant layers and the connective tissue immediately adherent to the mesothelium) is relatively unimportant as a physical transport barrier and does not provide a major limitation to small solutes permeability and osmotic ultrafiltration in PD [28]. In cases of alterations of the peritoneum over years, this conclusion may not apply.

Conventional dialysis solutions, containing high glucose lood, lead to formation of advanced glycation end-products (AGE) in peritoneum. In diabetic patients non-enzymatic glycation of proteins begins already in pre-dialytic period. Accumulation of AGE in peritoneum leads to increased permeability of peritoneal membrane. Diabetes mellitus, according to CANUSA studies, is more frequently related to high peritoneal permeability than other diseases causing renal insufficiency [29]. Diabetic patients show higher peritoneal transport, because AGE accumulation in peritoneum and in dialysate effluent is significantly greater in diabetics than in non-diabetics. Differences in peritoneal transport between insulin-dependent patients and insulin-nondependent ones were not demonstrated [30]. However, not all authors confirm more frequent occurrence of high transport in diabetics compared to non-diabetics [31,32]. Dimkovic et al. [33] showed even a greater number of low transporters among Asian Indian patients with diabetes than in non Indian ones. Recent study, using PDC [13], demonstrated that peritoneal function, including peritoneal membrane transport and peritoneal permeability to protein, was significantly higher in diabetics than in non-diabetics [34]. Therefore, hypoproteinemia in PD diabetic patients might be associated with high permeability of peritoneal membrane [34].

In 2003, PD adequacy parameters were related to histologic changes of peritoneal membrane, shown in a peritoneal biopsy at initiation of PD treatment and after a mean of 4 years on continuous ambulatory PD [35]. The main histologic changes were loss of mesothelial cells and decrease in normal mesothelial surface, thickening of the submesothelial collagenous zone, and presence of vascular hyalinosis. Only a trend was observed toward more severe lesions in patients treated with PD for about 4 years than in those starting PD. These not significant structural changes were not followed by functional changes during the first 4 years on PD. However, this study is limited by the small number (n = 18) of patients included [35].

After numerous experimental investigations, clinical trials with glycosaminoglycans have been already started to slow the progressive reduction in the dialytic efficiency of the peritoneal membrane. Intraperitoneal use of hyaluronan for 6-hour dwell in PD patients did not reveal significant changes neither in ultrafiltration nor peritoneal transport, but there was no adverse events related to hyaluronan administration [36]. Further studies with oral treatment of long-term PD patients with the glycosaminoglycan sulodexide showed that this drug improves some functional peritoneal membrane parameters (induces an increase in D/P urea and D/P creatinine and a decrease in peritoneal albumin loss), but it is inclear whether this therapy may be a strategy effective in stopping peritoneal dialytic failure [37].

New therapeutic strategies aiming to protect the peritoneal membrane from the consequences of long-term PD include [38,39]:

1. administration of L-arginine analogues,
2. modulating angiogenesis using agents that inhibit endothelial cell growth, adhesion and cell migration, or that interfere with vascular growth factors VEGF and βFGF, or their receptors,
3. gene therapy

   a) peritoneal mesothelial cells or peritoneal leukocytes can be modified to express antiinflammatory cytokines, as IL-1 receptor antagonist, the soluble receptor to TNF-α and IL-10,

   b) membrane integrity could be preserved enhancing the expression of fibrinolytic factors (tissue plasminogen activator) and anti-fibrotic molecules that counteract VEGF action and inhibit factor Kappa B and transforming growth factor β.

New aspects of the use of PD solutions alternative to glucose ones

Malnutrition is common among PD patients. Numerous factors lead to depletion of body tissue and nutrients, among them reduced nutrient intake, reflecting disturbed appetite, was recently proved in PD patients using electronic appetite rating system [40].

Amino acid-based dialysis solution (AA-DS) was designed to replace transperitoneal losses of amino acids and proteins during PD, thereby improving PD patients' nutrition. After many correction, AA-DS exerts beneficial nutritional effects, including improved nitrogen balance, increased concentration of plasma proteins, improved anthropometric measurements and improved plasma amino acid pattern [41,42]. However, Brulez et al. [43] have observed already in 1999 that absorption of L-methionine from AA-DS induces an increase in the plasma level of homocysteine by about 40% after use of AA-DS for 2 months and, therefore, increases the potential risk of cardiovascular illness. Perhaps AA-DS formulation can be still optimised. Recent data also demonstrate that AA-DS increases serum homocysteine level irrespective of patient sex, age, underlying disease, or diabetic status, and suggest that L-methionine content in AA-DS should be lower than 85 mg/dL, but optimal concentration was not established as yet [44].

It was shown in 2001 that leptinemia of patients switched to icodextrin dialysis solution is lower compared to that of a control group continuing to receive treatment with a glucose – based solution only [45]. Further studies of the same authors revealed that icodextrin administration leads to an increase in leptin peritoneal clearance, presumably as a consequence of increased ultrafiltration [46]. However, the previously established decrease in leptinemia during long-term icodextrin treatment cannot be simply an effect of an increased icodextrin peritoneal clearance. A role in the decrease in leptinemia during icodextrin treatment could also be played by reduced leptin synthesis following the decrease in glucose load and/or hyperinsulinemia and body fat mass in the long run [46].

Episodes of icodextrin-associated sterile peritonitis in patients maintained on chronic PD have been repeatedly described. In many cases this syndrome is caused by contamination of icodextrin with a gram-positive bacteria-derived peptidoglycan, a nonendotoxin pyrogen capable of provoking the inflammatory response in peritoneum. It is established that only a < 10 ng/ml peptidoglycan level fluid should be used, but some patients may become hypersensitive to the peptidoglycan contaminant in icodextrin solution. Such patients may only be able to tolerate fluid containing no peptidoglycan [47,48]. In contrast to bacterial peritonitis, there is no increase in CD14 (receptor for lipopolysaccharide) expression on the peripheral and peritoneal macrophages on the day of presentation and during the follow-up period of icodextrin-associated peritonitis [49].

Indications for use of peritoneal catheters with different configurations

With development of PD treatment, different types of peritoneal catheters are commercially available. Precise indications for their choice for individual patient are not well established. Chinese group of clinical investogators have published in 2003 the results of a prospective randomized controlled trial in PD patients who received a conventional straight double-cuffed catheter, a swan-neck straight catheter, or a swan-neck curled tip catheter [50]. These three different types of PD catheters did not have markedly different outcomes. However, there was a trend toward lower risk of exit site infections in swan-neck catheters, and significantly fewer swan-neck catheters were removed because exit site infections. The main benefit of swan-neck catheters was found in nasal non carriers of Staph. aureus, but swan-neck curled tip catheters had a high migration rate. Swan-neck straight catheters are therefore recommended by authors as the first-line catheters of choice, particularly in populations with a low rate of Staph. aureus nasal carriage.

Due to frequently observed migration of intraabdominal part of Tenckhoff catheter, techniques for correction of its position in the peritoneal cavity are continuously elaborated. Recently, the double guidewire method was introduced by Taiwan group [51]. The first guidewire is used to correct the direction of the catheter tip and the second guidewire is used to anchor the catheter so that an ideal course of the catheter can be maintained during removal of the first guidewire.

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Role of endothelial progenitor cells in cardiovascular pathology

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Abstract

Replacement of injured endothelial cells by bone marrow derived endothelial progenitor cells (EPC's) is a new pathway of vascular repair after ischemia. Endothelial progenitor cells contribute less than 0.01% to the peripheral venous compartment of mononuclear cells. The detection of EPC's requires a demonstration of CD 34 and VEGFR-2 (vascular endothelial growth factor receptor-2) antigenic cell membrane determinants and proof of endothelial characteristics after outgrowth and differentiation in cell culture. The most important stimuli to the mobilization and proliferation of EPC's are VEGF, GM-CSF (granulocyte-macrophage colony stimulating factor), erythropoietin, HMG-CoA-reductase inhibitors and tissue ischemia. In vivo in patients EPC's appear to contribute to endothelialization of vascular grafts, the formation of collaterals of ischemic limbs and the healing of myocardial infarcts. The role of EPC's in uremia is currently under investigation.

Key words: endothelial progenitor cells, vascular endothelial growth factor, erythropoietin.

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Introduction

Arteriosclerotic cardiovascular disease is a principal cause of death in the industrialized countries. An accelerated form of arteriosclerosis is found in patients with renal insufficiency or with end-stage renal failure. Endothelial injury is believed to be the first step in arteriosclerosis [1]. Endothelial injury is characterized by endothelial dysfunction, i.e. ineffective vascular dilatation in response to acetylcholine, thrombogenicity, enhanced adhesiveness of the endothelial surface and a loss of antiproliferative control over the adjacent layer of vascular smooth muscle cells [1]. It is likely that dysfunctional endothelial cells may recover upon cessation of the injurious insult(s) or they may be replaced by outgrowing endothelial cells from their neighbourhood. However, there may be an additional pathway: it was recently observed in recipients of a kidney transplant that the endothelial cells of capillaries in the graft were partly recipient derived, especially after an episode of rejection [2]. The cells were held to be derived from the recipient's bone marrow [2]. This and other observations opened up a new range of possibilities in endothelial regulation and repair. Much current research is devoted to the steps presumed to occur between the bone marrow stem cells and the vascular periphery as well as to the actual roles that bone marrow derived progenitor cells may assume in a mature endothelium.

Endothelial progenitor cells

Adult bone marrow contains (rare) pluripotent stem cells as well as stromal cells. Both cell types can be induced by activation of matrix metalloproteinase-9 to generate angioblast precursor cells, which in turn give rise to endothelial progenitor cells (EPC's) [3]. Asahara et al. [4] were the first to demonstrate the presence of EPC's in human peripheral blood. On the basis of known antigenic determinants found on cells involved in embryonic vasculogenesis – CD 34 and VEGFR-2 (vascular endothelial growth factor receptor-2); they were able to select

Table 1. **Marker proteins used in the differentiation of various endothelial progenitor cells (EPC's) and mature endothelial cells (EC's)**

	Bone marrow EPC's	Peripheral blood EPC's	Mature EC's
CD 133	+	+	-
CD 34	+	+	+
CD 31	-	+	+
VEGFR-2	+	+	+
VE Cadherin	-	+	+
vWF	-	+	+
CD 146	-	-	+

Abbreviations: CD 31: PECAM; vWF: von Willebrand factor; VEGFR-2: vascular endothelial growth factor receptor-2; Table modified after [4,10,23].

a fraction of cells from peripheral blood mononuclear cells that differentiated into endothelial cells under the conditions of cell culture – hence the term endothelial progenitor cells (EPC's). In addition the authors demonstrated homing of EPC's to sites of trauma and angiogenesis in vivo [4]. Reyes et al. [5] reported studies in vivo of bone marrow from healthy volonteer donors in which they showed the presence of stem cells within the samples and the subsequent differentiation of such stem cells into EPC's in response to cultivation in the presence of VEGF (vascular endothelial growth factor). Again, these EPC's contributed to wound healing in vivo later. Using subjects that had received a bone marrow transplant Lin et al. [6] also provided evidence of (donor) bone marrow derived EPC's in peripheral venous blood of recipients. In addition these authors showed a remarkable potential of their EPC's to multiply in culture. Over a time of 4 weeks they had a 1023-fold expansion of endothelial cells derived from EPC's [6].

Detection of endothelial progenitor cells

While it is clear that bone marrow stem cells give rise to EPC's which then leave the bone marrow, traverse the vascular compartment and home to the vascular endothelium of individual organs or tributaries there is debate on the surface markers to be used in the definition of EPC's. Most authors are in agreement that cells from the monocytic fraction of peripheral venous blood exhibiting CD 34 and VEGFR-2 antigenic determinants qualify as EPC's, i.e. as cells that later differentiate into mature endothelial cells and multiply prodigiously (*Tab. 1*). However, CD 133, VEGFR-3 and fibroblast growth factor receptor 1 have been suggested as additional characteristic surface markers of EPC's in some studies [7]. All of these marker proteins were originally known as antigens of embryonic hematopoetic stem cells and hematopoetic progenitor cells.

In order to study the number and function of EPC's it is customary to count circulating EPC's by FACS analysis. Their numbers in the peripheral circulation are very small. EPC's account for roughly 0.01% of circulating mononuclear cells. A second approach is to propagate them in culture [8]. The latter is used to determine their proliferation rate, their migratory capacity, their ability to form primitive tubes (termed: angiogenesis assay), their adherence function, and other qualities [8]. Cultured EPC's tend to lose their early progenitor markers – such as CD 133 – and aquire characteristics of endothelial cells – such as uptake of acetylated LDL, staining with Ulex Europeus agglutinin, expression of VE-cadherin and of CD 31.

Regulation of endothelial progenitor cells

The mobilization of EPC's from bone marrow and their circulating numbers are influenced by endogenous and exogenous factors as well as by pathological changes. The angiogenic growth factor VEGF has been shown in many studies to correlate with EPC counts and with EPC incorporation into sites of endothelial repair [9]. Injection of recombinant VEGF 165 in mice induced a rapid mobilization of hematopoetic stem cells and of EPC's, while a neutralizing monoclonal antibody to the VEGF-receptor-2 (VEGFR-2] inhibited these responses [9]. Other angiopoetic growth factors stimulating EPC mobilization are the following ones: angiopoietin-1, fibroblast growth factor, stroma cell derived growth factor-1 and placental growth factor [10]. 'The cytokines G-CSF and GM-CSF and the chemokine stem cell factor were also shown to increase EPC proliferation [10]. Bahlmann et al. studied the effects of erythropoietin in vivo in patients with moderate renal insufficiency and renal anemia [11]. They found a three-fold increase in the number of circulating EPC's together with a large augmentation of the proliferation rate in culture occurring within 2-6 weeks after the start of erythropoietin therapy [11].

Amongst exogenous factors HMG-CoA reductase inhibitors (statins) were shown to augment the numbers of hematopoietic stem cells in the bone marrow of mice and the proliferation rate of the corresponding EPC's in culture [12], effects that were mediated by the protein kinase Akt. The effects of statins were held to be at least as potent as those of VEGF [12]. Vasa et al. [8] extended these kinds of observations to human patients with coronary artery disease. Four weeks of therapy by 40 mg of atorvastatin daily increased the numbers of circulating EPC's threefold, augmented their proliferation rate in culture approximately fourfold and improved their migratory ability significantly [8].

Amongst pathological conditions with effects on bone marrow stem cells and EPC's ischemia has been demonstrated to provide a major stimulus [13]. Acute myocardial infarction in human patients, limb ischemia or vascular trauma during coronary artery bypass grafting were all associated with a rapid increase of EPC's in the circulation [14]. On the other hand there may also be downregulating effects at work in certain circumstances [15]. Studying 45 patients with stable coronary disease and 15 healthy volonteers, Vasa et al. [15] showed significant reductions of circulating EPC's and of the proliferation rate of EPC's in the patients with coronary artery disease. Of note they were able to correlate the number of atherosclerotic risk factors with the reductions of EPC

counts. In their study, smoking, a positive family history for coronary artery disease and hypertension were the major negative regulators of EPC's. The authors speculated that apoptosis was the mechanism involved in the observed effects. In another report – involving mice – it was suggested that age may lead to endothelial progenitor cell exhaustion and hence arteriosclerosis [16].

Potential roles of endothelial progenitor cells

We shall not address the issue of homing of EPC's in this communication. Assuming that there are ways to accomplish homing of EPC's to sites of involvement the question is: what purposes might be served by EPC's?

EPC's may contribute to rapid endothelialization. It was found in human patients that the surfaces of left ventricular assist devices had been colonized with CD 133+/VEGFR-2+ endothelial cells. Comparable observations have also been made on the internal surfaces of Dacron grafts in the aortic position of bone marrow transplanted dogs.

Werner et al. induced carotid artery injury in bone marrow transplanted mice resulting in the formation of a neointima [17]. They could show that bone marrow derived EPC's were involved in reendothelialization. However, in the presence of treatment by a HMG-CoA-reductase inhibitor the circulating pool of EPC's was enhanced, the homing of EPC's to the injured vessel wall was increased, reendothelialization was accelerated and neointima formation lessened [17].

Urbich et al. [18] studied neovascularization in a model of ligation induced hind-limb ischemia in the mouse. They gave intravenous infusions of EPC's that had undergone previous culture using VEGF or that were freshly isolated from blood. Only the former condition (culture and pretreatment with VEGF) yielded functionally improved neovascularization and augmented capillary density with demonstrable integration of EPC's into the respective vessels [18].

Glomerular endothelial cell injury and its repair in the Thy-1.1 model of the rat has also been studied [19]. In these observations an allogenic bone marrow transplant model was used to permit tracing of bone marrow-derived cells by MHC class I specific mAB. It was found that the damaged glomerular endothelium was repopulated by bone marrow-derived EPC's [19].

Potentially a very important area for EPC treatment may be coronary artery disease. Kamihata et al. [20] implanted EPC's in a porcine myocardial infarction model. Three weeks after implantation the treated animals had improved regional blood flow, increased capillary density and a higher number of collateral vessels at the infarct zone than control animals [20].

In a recent study in humans after acute myocardial infarction 20 patients received an infusion of autologous progenitor cells into the infarct artery [21]. Several months later the treated group was reported to show a significant increase in left ventricular ejection fraction and improved regional wall motion in the infarct zone as compared to untreated controls [21]. Another study in human patients was able to confirm these results [22]. Three months after intracoronary application of autologous progenitor cells the treated patients had a significantly decreased infarct region and a significant increase of myocardial wall motion in the infarct region compared with control.

In moderate renal insufficiency mildly reduced counts of circulating EPC's have been demonstrated [11]. Ongoing work in our own group serves to delineate the functional properties of EPC's in patients with uremia undergoing treatment by hemodialysis.

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Continuous modalities of renal replacement therapy. Review of selected aspects

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Abstract

Severe clinical course of acute renal and acute liver failure is recognized as life-threatening condition, often developing in critically ill patients, demanding intensive care. Continuous renal replacement techniques, including veno-venous hemodialysis/hemofiltration/hemodialfiltration/ /ultrafiltration (CVVHD/CVVHF/CVVHDF/SCUF) are becoming the therapeutic modality of choice in unstable patients with acute renal failure. Recently introduced new technique – albumin dialysis (MARS – Molecular Adsorbent Recirculating System) is a very useful extracorporeal method of detoxication in patients with acute liver or kidney/liver failure. It may also serve as bridge to liver transplantation in patients with rapidly deteriorating chronic liver failure. Indications, benefit/risk ratio and specific clinical and technical aspects are briefly described in this review paper.

Key words: continuous hemodialysis-hemodiafiltration, albumin dialysis, MARS.

Continuous renal replacement therapy (CRRT)

Acute renal failure in unstable patients is the main indication for continuous renal replacement techniques (CRRT). There are four types of these modalities, including veno-venous hemodialysis/hemofiltration/hemodialfiltration/ /ultrafiltration (CVVHD/CVVHF/CVVHDF/SCUF). The arterio-venous variant of hemofiltration is now abandoned in most centres, due to technical and clinical superiority of veno-venous techniques [1,2]. Advantages and contraindications are summarized in *Tab. 1*.

Standard vascular access required to perform veno-venous CRRT techniques is regular double-lumen hemodialysis catheter, introduced to the central vein by Seldinger technique or (in specific cases) surgically. Currently available CRRT machines offer advanced electronic control of several technical parameters, crucial for efficacy and safety of the procedure and friendly for the medical staff. The blood flow driving force is almost independent from the patients current blood pressure values and both ultrafiltration/supplementation rates planned by physician are actively created and monitored by machine. Fully integrated electronic system adjusts all the required parameters to desired balance.

Depending on specific clinical conditions – different techniques are used, with the same device. The options are summarized in *Tab. 2*.

In terms to follow new clinical requirements, the initially introduced technique may be extended. If e.g. patients treated by SCUF develop uremic toxicity, the modality may be extended to CVVHD or CVVHDF, simply by adding supplementation or dialysate flow. The higher flow of these fluids – the higher efficacy of detoxication. The range of fluid flow rates depends on patients body weight (volume distribution) and severity of uremic toxicity. Both (supplement and dialysate) may be given with velocity from 100 to 1500 ml/h. Commercially available fluids contain ions and buffers in concentration resembling physiological plasma water. The exception is potassium, which (depending on current kaliemia) must be added to the fluid, if required [3-7].

According to several reports, potentially advantageous effect of CVVHF in septic patients is based not only on uremic toxins/water elimination, but also cytokines (range of 10000--50000 daltons) and bacterial endotoxins removal. Tumor

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***Table 1*. Continuous renal replacement techniques in acute renal failure (ARF)**

Modality	Main indications	Contraindications	Advantages	Disadvantages Common complications
CVVHF CVVHD CVVHDF SCUF	– ARF with massive overhydration – ARF in nephrotic patients – parenteral nutrition in anuric patients – ARF in patients with congenital defects or surgery-related damage of abdomen cavity and wall (contraindication for peritoneal dialysis) – ARF with extreme BUN concentration and coma (high risk of brain oedema)	– poor quality of vascular acess * – severe coagulation defects ** – severe active bleeding, especially to CNS ** * relative problem, solved by change of access ** relative problem, solved by reduction or withdrawing of heparinisation	– gentle, slow detoxication – well, automatically controlled ultrafiltration rate – performable in unstable patients with heart failure, or with septic shock, remaining on catecholamines	– problems with maintaining vascular access with prolonged therapy – thrombocytopenia with prolonged heparinisation – high dialysate consumption in hypercatabolic cases – long immobilization of the patient with all side-effects of this condition

***Table 2*. Specific indications for different CRRT modalities**

Specific indication	Recommended CRRT modality
Overhydration, low uremic toxicity (e.g. ARF in nephrotic patients)	SCUF
ARF in septic patients (potential advantage of cytokines removal)	CVVHF/CVVHDF
High BUN, hypercatabolic patients, parenteral nutrition	CVVHD/CVVHDF

necrosis factor (TNFα), interferon-γ and several interleukins (IL-1, -2, -6) are considered candidates. The reliability of this aspect is controversial, as no extended, controlled studies are available [8,9].

As all extracorporeal techniques, CRRT modalities require heparinisation, applied up to personal experience. If non-fractioned heparin is selected – bolus dose of 0.3-0.5 mg (30-50 u.)/kg/dose, followed by continuous infusion of 0.1-0.2 mg (10-20 u.)/kg/h is recommended. Activated coagulation time (ACT) must be monitored, to maintain a target range of 170-200 s. Low molecular-weight heparin (LMWH) iv., at repeated doses of 50-100 j.antyXa./kg/6 h may be administered alternatively. Thrombocytopenia is specific complication in patients treated with CRRT during several, following days. Obviously, in patients at bleeding-risk the heparinisation should be reduced or withdrawn.

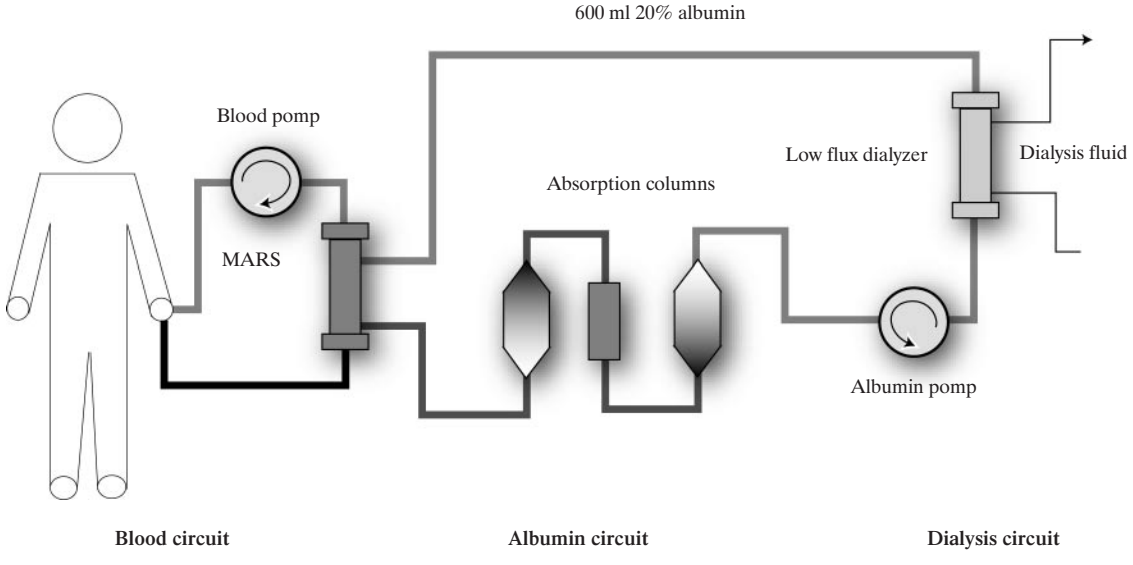
Continuous liver-renal replacement therapy Albumin dialysis – MARS (Molecular Adsorbent Recirculating System)

Indication for MARS therapy is liver failure with hepatic encephalopathy and liver-kidney failure in cases of hepato-renal syndrome [10,11]. Hepatic encephalopathy is a neuropsychiatric syndrome occurring in several patients with both acute and chronic liver failure. It is characterized by altered sleep-wake cycle, confusion and disorientation, asterixis, hyperreflexia and slowing of the dominant rhythm on electroencephalography. It is potentially reversible, if liver function improves, spontaneously or under specific treatment and in cases, when the liver transplantation is successfully performed on time. Hepatic encephalopathy usually develops slowly in chronic, cirrhotic patients, but in cases of acute liver failure the onset is sudden and it may progress very rapidly. Coma and brain herniation are the main life-threatening complications. Encephalopathy is one of the major determinants of very high mortality and irreversible brain damage in acute liver failure. The pathogenesis of hepatic encephalopathy is multifactorial. One of the mechanisms is the systemic accumulation of several neuroactive and neurotoxic substances, normally metabolised and cleared by the liver. Massive astrocytes swelling is the cause of brain oedema and, if is not overcome, fatal brain herniation. Arterial ammonia concentration seems to correlate with occurrence of increase of intracranial pressure and cerebral herniation. Other important substances include cerebral benzodiazepines, serotonin, manganese, inflammatory cytokines and bilirubin. Many of these substances, as bound by plasma albumins, are water-insoluble. Extracorporeal removal of liver toxins is the target of specific therapeutic modalities. The aim of such therapy is either to avoid hepatic encephalopathy or ameliorate existing condition, while awaiting spontaneous improvement of hepatic function or liver transplantation. It must be stressed, that urgent liver transplantation is the optimal therapeutic option for patients with hepatic encephalopathy, unless irreversible brain damage occurs [12].

Recently a new method designed for the selective removal of albumin-bound substances MARS (Molecular Adsorbent Recirculating System), originally developed by Dr Jan Stange and Dr Steffen Mitzner from University of Rostock (Germany), became available [13-17].

The aim of MARS technology is continuous selective

Figure 1. **MARS – circulating systems**



removal of small and medium sized molecules from blood. The special polysulfone high-permeability MARS membrane absorbs lipophilic, protein-bound toxin from patient's blood onto one side and is simultaneously cleaned by selective molecular adsorbents (albumin dialysate) from the other side. Small – sized and water – soluble substances are also dialyzed through this membrane, as during conventional hemodialysis. Therefore MARS therapy enables specific simultaneous elimination of the liver failure albumin – bound toxins and water – soluble uremic toxins, using "intelligent" membrane transport [18].

Technical aspect of the procedure is similar to all other extracorporeal modalities. The patient's blood flows through a catheter via an extracorporeal circuit into blood compartment of capillary MARS-flux dialyzer. The outside of MARS membrane is cleansed by a recirculating human albumin 20% solution (albumin "dialysate"). As the "liver toxins" are transported by bounding proteins, this mechanism produces the driving force across the MARS membrane. Albumin dialysate is then regenerated in closed circuit. It flows through the blood compartment of the second dialyzer (DIA-flux) and undergoes regeneration by bicarbonate-buffered dialysate in open-loop, single pass dialysate circuit ("renal detoxification" – water and small soluble toxins are removed). On the next step the albumin dialysate is passing through two sequential columns; the first containing uncoated charcoal to bind non-ionic toxins and the second – an anion exchanger resin to remove the toxins still bound to albumin ("hepatic detoxification"). After this regeneration purified albumin dialysate returns to clean MARS membrane in closed circuit. The MARS Monitor is connected to the hemodialysis machine, allowing continuous flow of albumin dialysate through dialyzer MARS-flux and its regeneration in DIA-flux dialyzer and two adsorbers. It can cooperate with majority of standard dialysis machines and machines for continuous renal replacement therapy (CRRT) [18].

MARS procedure effectively removes several substances from the patient's blood: albumin bound – bilirubine, bile acids, aromatic amino acids, benzodiazepin like substances, short and middle chain fatty acids and water soluble – ammonia, creatinine, urea, copper, iron. The procedure is usually performed in continuous manner – the time of single session ranges from 8 to 24 hours. The absorbing capacity of columns decreases with time, so depending on basic concentration of toxic substances – the circuit must be exchanged, once the columns are saturated. Slow and gentle removal of several toxins improves the clinical safety, especially in cases at risk of brain herniation. Patients with severe coagulation defects require no heparinisation during MARS procedure, however with gradual improvement of liver insufficiency – ACT must be checked regularly and unfractioned heparin should be added to the circuit, to maintain ACT within the range 170-200 s.

MARS therapy has a positive impact on patient's survival, systemic hemodynamics, neurological status, course of hepatic encephalopathy, cerebral blood flow velocity, intracranial pressure, intrahepatic cholestasis, kidney and liver function, toxin load, albumin binding capacity, electrolyte and acid-base balance.

It must be stressed, that MARS therapy should be used by specialized centers, able to proceed all the steps of management, required by critically ill patients with liver or multiorgan failure [19,20].

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Urinary tract infection – 2003

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Abstract

Urinary tract infections (UTI) are one of the most common and most intensively studied infections encountered in clinical practice. It is, however, an area where significant confusion has existed in agreeing the criteria for diagnosis, the natural history of disease, and the treatment.

In the present paper etiology and risk factors for uncomplicated UTI, treatment of UTI, estrogen in UTI treatment in postmenopausal women and UTI in patients with diabetes and renal insufficiency were discussed.

Key words: urinary tract infection, risk factors, etiopathogenesis, diabetes, asymptomatic bacteriuria.

Uncomplicated, community-acquired urinary tract infections (UTI) in women

Discussion of uncomplicated urinary infection primarily relates to a condition affecting women. It has been estimated that most women will experience at least one urinary tract infection during their lifetime. Uncomplicated UTI may involve the bladder or the kidneys and may be symptomatic or asymptomatic.

Most acute lower UTI (also termed acute bacterial cystitis) are uncomplicated – that is, they are not associated with structural abnormalities of the urinary tract, diabetes, immunosuppression, pregnancy, previous pyelonephritis, or symptoms lasting more than 14 days.

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After an initial infection, most women have sporadic recurrences, and a quarter to half have another infection within one year [1].

Etiology

Escherichia coli causes 75 to 90 percent of episodes of acute uncomplicated cystitis, and Staphylococcus saprophyticus accounts for 5-15%, mainly in younger women. Enterococci and aerobic gram-negative rods other than E. coli, such as Klebsiella sp. and Proteus mirabilis, are isolated in the remainder of the cases [2]. E. coli that encode the type 1 pilus, an organelle containing the adhesin FimH, which recognizes a wide range of cell types, are commonly associated with cystitis as well as sepsis and meningitis [3].

Considerable evidence supports the concept that the initial event leading to community-acquired UTI is intestinal colonization with a uropathogenic strain of E. coli. Once colonization has occured, the strain may remain part of the colonic flora for months, whether or not it causes a UTI. Its persistence in the colonic flora is facilitated by the same bacterial adhesins that promote attachment to the uroepithelium [4].

Risk factors

Women who experience acute UTI are characterized by both a genetic predisposition and behavioral factors. The most exposed are non-secretors of blood group substance and first degree female relatives with recurrent urinary infections. The most important behavioral risk factors are: recent sexual activity, use of spermicidal agents and diaphragm. Other behavioral factors include: frequency of urination, aspects of personal hygiene or use of the birth control pill. Despite the frequency of acute uncomplicated UTI, there is little long-term morbidity and no evidence for mortality attributable to this problem [5]. These women are not at increased risk of developing hypertension or renal failure. Short-term morbidity due to acute symptoms may however be substancial, especially for women with frequent UTI.

Table 1. **Antimicrobial regimens for prevention and treatment of recurrent urinary tract infections [8].**

Antimicrobial agent	Dosage for treatment	Daily dosage for prevention
Cefalexin	500 mg three times daily for three days	125 mg
Ciprofloxacin	100 mg twice daily for three days	125 mg
Amoxiclav	375 mg thrice daily for three days	No data available
Co-trimoxazole	960 mg twice daily for three days	240 mg (or three times a week)
Nitrofurantoin	50 mg four times daily for seven days	50-100 mg
Nitrofurantoin macrocrystals and monohydrate	100 mg twice daily for seven days	100 mg
Norfloxacin	200 mg twice daily for three days	200 mg
Trimethoprim	200 mg twice daily for three days	100 mg (or three times a week)

Treatment

In some locales, such as the southeastern and western United States and in Poland, resistance to trimethophrim-sulfametoxazole has become widespread and is detected in up to 20-25% of the pathogens cultured from the urine of women with acute cystitis, most commonly those who have received this agent within the preceding six month [5,6]. The prevalence of such resistance varies not only from country to country, but even from hospital to hospital [7].

The alarming reports of community-acquired UTI caused by fluoroquinolone-resistant E. coli strains in some parts of the world suggest that we will see an evolution of resistance to these agents just as we have with sulfonamides, ampicillin, oral cephalosporins, and now trimethoprim - sulfamethoxazole unless we take a much more aggressive approach to the control of antimicrobial resistance [4].

Nevertheless most of authors maintain that the first choice of treatment is trimethoprim, except in women from communities with a high rate of resistance, when one should follow the local guidance. The main alternatives are: norfloxacin, amoxiclav and nitrofurantoin. Antimicrobial regimens for treatment and prevention of recurrent UTI are enlisted in *Tab. 1*. A three day course of antibiotic treatment should suffice for most women with lower UTI. If, despite treatment, symptoms persist or worsen, urine culture should be performed and antibiotics according to the results of the culture and sensitivity tests should be prescribed. Upper UTI in otherwise healthy women can be treated with oral antibiotics for 7-10 days. Women who are systemically unwell should be admitted to hospital [8].

Women who have frequent recurrences (arbitrarily defined as three or more infections a year) should be advised to avoid exposure to vaginal spermicides and should be offered prophylaxis or methods of self-treatment. Imaging studies should be reserved for women with complicated infections [5].

Interestingly, there is some evidence that cranberry juice treats UTI and prevents its recurrence [9].

Exogenous estrogen in preventing recurrent UTI in postmenopausal women

It has been hypothesized that exogenous estrogen can prevent recurrent cystitis by reversing genitourinary mucosal atrophy and restoring a more normal milieu in the vagina. In a randomized, open-label study, the use of an estrogen–impregnated ring, or topical estriol cream were associated with a significant reduction in recurrent infections [10], but further studies with larger sample sizes are needed.

Although small studies have suggested a benefit associated with oral estrogen replacement therapy, recent randomized trials have failed to show a favorable effect in preventing cystitis, and there is currently no rationale for prescribing oral estrogens to prevent recurrent cystitis [11].

Urinary tract infections in patients with diabetes mellitus and renal insufficiency

Diabetic patients with renal disease are at increased risk of developing urinary tract infections due to functional complications of the urinary tract, impaired host defence mechanisms, and poor urinary flow. Interestingly, the frequency of UTI has been shown to be increased only in diabetic women compared with non-diabetic women, but not in diabetic men compared with non-diabetic men. This difference may be secondary to the increased incidence of vaginitis among women with diabetes mellitus. UTI in these patients may be further complicated by pyelonephritis, intrarenal or perinephric abscesses, papillary necrosis, and sepsis. In addition, the wide spread use of broad-spectrum antibiotics in recent years has selectively causes an increased incidence of fungal infections in the urinary tract [12].

Pathogenesis

Microorganisms may infect urinary tract by ascending through the urethra into the bladder or by haematogenous or lymphatic spread. The first mechanism is by far most common and can explain the association of the increased incidence of urinary tract infection in patients with frequent vaginitis and lower urinary tract abnormalities. Reported antibacterial host factors include the urea, organic acids, and salt content of the urine, and a high osmolality in the presence of the low pH. Other factors protecting the host include urinary inhibitors to bacterial adherence such as Tamm-Horsfall protein (THP), bladder mucopolysaccharide, low-molecular-weight oligosaccharides, secretory IgA and lactofferin.

In addition, proper urine flow, micturition and emptying of the bladder are crucial in inhibiting bacterial proliferation and extension to the upper urinary tract.

Role of Tamm-Horsfall protein in pathogenesis of UTI

There are some interesting informations concering the role of THP in the kidney defence against UTI, particularly those caused by E. coli.

Pak et al. [13] recently showed, that THP binds type 1 fimbriated E. coli in vitro and efficiently competes with uroplakin Ia and Ib in binding to these pathogens. These results support the notion that in vivo, urinary THP represents a protective agent against UTI, because type 1 E. coli strains represent the predominant phenotypic variants of isolates from patients with UTI, and uroplakin Ia and Ib behave as efficient cell receptors for type 1 fimbriated E. coli [14].

On the other hand it is known, that glication of THP in patients with diabetes, or in renal diseases can changes its ability to inhibition of bacterial adherence to human uroepithelial cells.

Asymptomatic bacteriuria in diabetics

The incidence of asymptomatic bacteriuria in diabetic patients is estimated from several studies to range between 7 and 32%. Despite its high incidence in diabetic patients, asymptomatic bacteriuria is not affected by hyperglycaemic control or the degree of renal failure [15]. Empirical treatment of asymptomatic bacteriuria still remains a clinical challenge. Undertreatment may theoretically predispose to pyleonephritis, renal papillary necrosis, and renal insufficiency, whereas overtreatment may give rise to multiple complications including poor tolerance to the side effects of antimicrobial agents, development of antibiotic-resistant organisms, fungal superinfections, and antimicrobial-induced renal failure. The latter complication may be irreversible in patients with underlying renal insufficiency. In diabetic patients, the presence of asymptomatic bacteriuria may be more problematic, especially in those with associated diabetic neurogenic bladder and urinary retention.

Treatment of asymptomatic bacteriuria requires careful clinical judgment. In general, the prognosis for asymptomatic bacteriuria is excellent. 14-year follow-up study of untreated asymptomatic bacteriuria in diabetic patients revealed similar frequencies of acute pyelonephritis, deterioration of kidney function, and systemic hypertension compared with control subjects [16].

Currently, treatment of asymptomatic bacteriuria is recommended for patients with frequent episodes of symptomatic UTI, pregnancy, after renal transplantation, and prior to urological interventions, but there are no substantial benefits from treating diabetics with asymptomatic bacteriuria [17].

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Renal affection in patients with diabetes mellitus is not always caused by diabetic nephropathy

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Abstract

Accumulated clinical data suggest that non-diabetic nephropathy complicating type 1 diabetes mellitus is rare, accounting for 2-3% of unselected diabetic patients with proteinuria. In contrast, non-diabetic kidney disease is a common finding in patients with type 2 diabetes mellitus. Joint analysis of available data on prevalence of non-diabetic kidney disease among type 2 diabetic patients revealed that non-diabetic nephropathy was evident on kidney biopsy approximately in 22% of European and 26.7% of Asian patients with type 2 diabetes mellitus. Therefore, kidney biopsy may become a useful diagnostic option among proteinuric patients with type 2 diabetes mellitus. However, it is generally agreed that renal biopsy cannot be used as a routine diagnostic test in all type 2 diabetic patients with proteinuria. Diabetic subjects that may benefit form kidney biopsy should be rather identified on a case-by-case basis. Absence of diabetic retinopathy, particularly used in combination with acanthocyturia, may come useful in decisions about kidney biopsy in type 2 diabetic patients.

Key words: diabetes mellitus, diabetic nephropathy, non-diabetic nephropathy, kidney biopsy.

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Kidney biopsy in diabetic patients with proteinuria – potential therapeutic implications

Proteinuria in diabetic patients is usually interpreted as a clinical manifestation of diabetic nephropathy [1]. Although kidney biopsy is the most unbiased method of evaluation in proteinuric patients, it is rarely used in subjects with diabetes mellitus. Theoretically, in each proteinuric patient with diabetes mellitus kidney biopsy may reveal different renal pathologies including:

a) diabetic nephropathy,
b) non-diabetic kidney disease superimposed on diabetic nephropathy,
c) non-diabetic nephropathy,
d) normal renal structure.

Consequently, therapeutic decisions based on the results of the biopsy may differ – treatment of diabetic patients with non-diabetic nephropathy (i.e. primary glomerulonephritis) may be adjusted to eliminate the primary trigger causing the renal pathology with a potential to halt or reverse the decline in renal function. Paradoxically, therapy in subjects whose renal structure was normal on kidney biopsy will not differ significantly from patients with biopsy-confirmed diabetic nephropathy – in both cases prevention of diabetic kidney disease is warranted. Thus, the primary aim of kidney biopsy in proteinuric patients with diabetes mellitus is to confirm/exclude non-diabetic renal disease.

Non-diabetic kidney disease in patients with type 1 diabetes mellitus

A routine kidney biopsy in proteinuric patients with type 1 diabetes mellitus, although postulated by several authors [2], is not supported by both prospective as well as [3,4] cross-sectional studies [5]. A long-term prospective observation of a large cohort of Caucasians have provided evidence for uncommon

occurrence of non-diabetic kidney disease among patients with type 1 diabetes mellitus – only 2.5% of subjects developed non-diabetic proteinuria [3]. A slightly higher rate (9%) of non-diabetic renal disease in patients with type 1 diabetes mellitus reported in a cross-sectional observation may be caused by a selection bias (inclusion of patients with atypical clinical manifestation of renal disease) [5]. It is generally agreed that non-diabetic nephropathy complicating type 1 diabetes mellitus is relatively rare, accounting for 2-3% in unselected proteinuric patients with diabetes longer then 10 years [6]. Although guidelines with precise indications for kidney biopsy in subjects with diabetes mellitus are lacking, accumulating clinical data suggest that renal biopsy should not be a routine diagnostic test in proteinuric patients with type 1 diabetes mellitus.

Non-diabetic kidney disease in patients with type 2 diabetes mellitus

Due to significantly higher prevalence of type 2 diabetes mellitus (when compared to type 1) as well as its increasing rates of incidence in many populations [7] chronic kidney disease in type 2 diabetic patients is common in clinical setting. In contrast to data on type 1 diabetes mellitus, there is no general agreement on prevalence of non-diabetic kidney disease among patients with type 2 diabetic mellitus. Consequently, it is not clear whether kidney biopsy should become a part of the standard evaluation of proteinuric patients with this type of diabetes mellitus.

In order to evaluate genuine rates of non-diabetic nephropathies in patients with type 2 diabetes, we analysed available English publications on kidney biopsy in proteinuric patients with this type of diabetes mellitus [8-18]. Altogether, 665 cases of renal biopsies were included in the analysis that was performed separately for Caucasian and Asian populations. Prevalence of biopsy-proven diabetic nephropathy, non-diabetic kidney disease superimposed on diabetic nephropathy, non-diabetic nephropathy and normal renal structure were reported for each study and the final average percentage of each kidney biopsy finding was calculated. The results of this analysis are presented in *Tab. 1*.

Prevalence of non-diabetic kidney disease among European patients with type 2 diabetes mellitus varied from 3% among Danish [8] to 32% in Italian subjects [12]. Coexistence of both non-diabetic nephropathy and diabetic nephropathy was the most infrequent result of kidney biopsies. On average, diabetic nephropathy was the most common pathology in proteinuric patients with type 2 diabetes mellitus (64.8%), followed by non-diabetic kidney diseases (18.7%), normal renal structure (13.2%) and non-diabetic nephropathy superimposed on diabetic nephropathy (3.3%). Altogether, non-diabetic kidney disease was present in approximately 22% of patients with type 2 diabetes mellitus.

Analysis of kidney biopsy results from Asian patients with type 2 diabetes mellitus (subjects from Japan, China and India) provided consistent findings. A significant variation in prevalence of diabetic nephropathy (from 87.7% in subjects from India [13] to 35.3% in Chinese patients [18]) was evident across the studies. Non-diabetic nephropathy without coexistent diabetic nephropathy was found in 16.8% cases and superimposition of chronic non-diabetic renal pathology on diabetic nephropathy accounted for 9.9% cases. Altogether, non-diabetic renal disease affected 26.7% of Asian subjects with type 2 diabetes mellitus.

Significant differences in prevalence of non-diabetic renal diseases among the studies are caused, at least in part, by discrepancies in methodology. First, in several studies [18] only diabetic patients with atypical clinical manifestation of renal disease were included in the study group. This may lead to overrepresentation of cases with non-diabetic kidney disease among proteinuric subjects with type 2 diabetes mellitus. Second, different pathologic criteria of diabetic nephropathy were used in several studies and this may result both in overestimation and underestimation of non-diabetic renal disease in diabetic patients. Finally, a confounding influence caused by interobserver variability cannot be excluded in several studies [19].

Taken together, the results of this analysis indicate that, even after adjusting for differences in methodology among the studies, non-diabetic renal disease may affect a significant percentage of patients with type 2 diabetes mellitus. Therefore, kidney biopsy may become a useful diagnostic option among proteinuric patients with this type of diabetes mellitus.

Types of non-diabetic kidney disease among type 2 diabetic patients undergoing kidney biopsy

Primary glomerulonephritis was the most common renal pathology among all types of non-diabetic kidney disease in patients with type 2 diabetes mellitus. Almost all types of glomerulonephritis including IgA nephropathy [11,15,19-20] membranous nephropathy [18], mesangiocapillary glomerulonephritis [18,20], rapidly progressive glomerulonephritis [14] and minimal change disease [15] were represented in these patients. In fact, coexistence of more then one type of glomerulonephritis, superimposed on diabetic nephropathy, although not common, was reported in diabetic subjects [21].

IgA nephropathy was consistently the most common type of glomerulonephritis in both Caucasian [11,19] and non-Caucasian [15] populations accounting for approximately 6-19% of kidney biopsies. Several observations suggest that common occurrence of IgA nephropathy in diabetic patients is not coincidental [22-24]. Diabetes itself, by affecting either glomerular structure and function [24] and/or non-enzymatic glycation of immunoglobulins may facilitate [16] the development of immunopathological alterations [24]. In support of this notion, circulating levels of IgA fraction of immunoglobulins were higher in type 2 diabetic patients than age- and sex-matched controls [15] and superimposition of IgA nephropathy on diabetic nephropathy was associated with higher IgA levels when compared to diabetic nephropathy alone [16]. Nevertheless, IgA nephropathy remains the most common type of glomerulonephritis in the general population [24] and its high prevalence among type 2 diabetic patients may simply reflect general epidemiological trends.

Tubulointerstitial renal disease was a relatively rare finding on renal biopsy in patients with type 2 diabetes mellitus [13,17]. In particular, pathology indicating chronic pyelonephritis was not common among diabetic patients undergoing kidney biopsy [13]. This under-representation of chronic pyelonephritis among type 2 diabetic patients is surprising in light of the well-known tendency to asymptomatic bacteriuria among diabetic subjects [25].

Clinical markers of non-diabetic kidney disease among patients with type 2 diabetes mellitus and renal affection

In light of relatively high prevalence of non-diabetic kidney disease among patients with type 2 diabetes mellitus indication for routine kidney biopsy was postulated by several authors [19]. However, it is generally agreed, that kidney biopsy cannot be applied as a standard diagnostic test in all type 2 diabetic patients with proteinuria and identification of subjects that may benefit from kidney biopsy should be made on a case-by-case basis. Several clinical and laboratory features including absence of diabetic neuropathy [26], absence of diabetic retinopathy [10], hematuria [15], short duration of diabetes [27], sudden progression of renal failure [11] and acanthocyturia [28] were proposed as markers of non-diabetic renal disease in diabetic patients. None of the proposed markers has either absolute sensitivity or 100% specificity for non-diabetic renal disease. Therefore, they cannot be used as sole indicators of non-diabetic renal disease in patients with type 2 diabetes mellitus. Nevertheless, some of these markers, particularly used in combination, may come useful when decisions about kidney biopsy in type 2 diabetic patients are made.

Absence of diabetic retinopathy as a marker of non-diabetic renal disease in patients with type 2 diabetes mellitus and renal affection

It is not clear to what extent absence of diabetic retinopathy in type 2 diabetic patients with renal affection may serve as an indicator of non-diabetic renal disease. To address this issue we performed a joint analysis of 4 available publications (*Tab. 1*) [11,19,27,29] evaluating prevalence of non-diabetic renal disease in type 2 diabetic patients without diabetic retinopathy. Altogether, 154 Caucasian diabetic patients undergoing kidney biopsy were included in this analysis. Consistent with the previous joint examination, there were substantial differences in prevalence of non-diabetic renal disease among the studies. As discussed before, these discrepancies are probably related to the lack of methodological consistency among these investigations. Overall, the prevalence of diabetic nephropathy, non-diabetic renal disease superimposed on diabetic nephropathy, non-diabetic renal disease and normal renal structure were 45.5%, 13.6%, 35.1% and 5.8%, respectively. Subjects with apparent non-diabetic renal disease accounted for 48.7% of type 2 diabetic patients without diabetic retinopathy. This number is almost twice higher when confronted with the percentage of cases of non-diabetic renal disease among type 2 diabetic patients with renal affection.

These results are supported by the data on prevalence of diabetic retinopathy among type 2 diabetic patients with diabetic nephropathy when compared to type 2 diabetic subjects with non-diabetic renal disease. Coexistence of diabetic retinopathy with biopsy-proven diabetic nephropathy was documented in 41.4-75% patients [11-20]. In contrast, diabetic retinopathy was consistently absent [10-11,30] or present in a small (9.7%) number of patients [18].

Altogether, these data suggest that diabetic retinopathy occurs significantly less frequently in type 2 diabetic patients with non-diabetic renal disease when compared to subjects with diabetic nephropathy. Therefore, absence of diabetic retinopathy in proteinuric patients with type 2 diabetes mellitus may suggest that non-diabetic renal disease may be responsible for renal affection.

Microscopic hematuria and acanthocyturia as markers of non-diabetic renal disease in patients with type 2 diabetes mellitus

Although microscopic hematuria has been suggested as a rare clinical finding in diabetes mellitus [31], its prevalence in diabetic subjects in most of the available studies was relatively high (from 29% [15] to 72.7% [32]). In addition, comparison of rates of microscopic hematuria between patients with diabetic nephropathy and primary glomerulonephritis revealed no statistically significant differences [18]. Taken together, these data indicate that utility of microscopic hematuria as a marker of differentiation between diabetic and non-diabetic renal disease is limited.

Urinary excretion of acanthocytes (dysmorphic ring-formed erythrocytes with vesicle-shaped protrusions) has been long recognised as a marker of glomerular bleeding [33]. Unlike other dysmorphic red cells (echinocytes, anulocytes, ghost cells, schizocytes, stomatocytes, knizocytes) found on urinalysis, clinically significant acanthocyturia (>5% of excreted erythrocytes) was shown as a relatively sensitive (approximately 52%) and very specific (98%) marker of primary glomerulonephritis [33]. The recent clinical study on acanthocyturia supports its utility as a diagnostic tool to differentiate diabetic kidney disease and glomerulonephritis – acanthocytes were significantly less frequent in urinalysis from patients with diabetic nephropathy when compared with subjects with biopsy-proven primary glomerulonephritis [24].

Conclusions

Non-diabetic kidney disease is a relatively common finding in patients with type 2 but not type 1 diabetes mellitus. Reliable confirmation of non-diabetic kidney disease in a diabetic patient with renal affection requires renal biopsy. It is reasonable to postulate that although renal biopsy cannot be a routine diagnostic procedure, it should be taken into consideration in all

Table 1. **Prevalence of non-diabetic kidney disease in type 2 diabetic patients undergoing renal biopsy**

Study No	Total number of subjects	Subjects with diabetic nephropathy	Subjects with non-diabetic renal disease superimposed on diabetic nephropathy	Non-diabetic kidney disease	Normal renal structure	Reference
Caucasian populations						
1.	33-100%	87.9% (29)	9.1% (3)	3.0% (1)	0% (0)	8.
2.	26-100%	84.6% (22)	0% (0)	15.4% (4)	0% (0)	9.
3.	35-100%	77.1% (27)	0% (0)	22.9% (8)	0% (0)	10.
4.	35-100%	74.3% (26)	8.6% (3)	11.4% (4)	5.7% (0)	11.
5.	53-100%	26.4% (14)	0% (0)	32.1% (17)	41.5% (22)	12.
Altogether	182-100%	64.8% (118)	3.3% (6)	18.7% (34)	13.2% (24)	
Non-diabetic kidney disease – altogether			22.0% (40)			
Asian populations						
1.	260-100%	87.7% (228)	1.2% (3)	11.1% (29)	0% (0)	13.
2.	35-100%	71.4% (25)	14.3% (5)	14.3% (5)	0% (0)	14.
3.	51-100%	66.7% (34)	17.6% (9)	15.7% (8)	0% (0)	15.
4.	53-100%	66.0% (35)	44.0% (18)	0% (0)	0% (0)	16.
5.	16-100%	50.0% (8)	0% (0)	50.0% (8)	0% (0)	17.
6.	68-100%	35.3% (24)	20.0% (13)	45.6% (31)	0% (0)	18.
Altogether	483-100%	73.3% (354)	9.9% (48)	16.8% (81)	0% (0)	
Non-diabetic kidney disease – altogether			26.7% (129)			
Type 2 diabetic patients without diabetic retinopathy						
1.	23-100%	73.9% (17)	0% (0)	26.1% (6)	0% (0)	11.
2.	49-100%	69.4% (34)	0% (0)	12.2% (6)	18.4% (9)	19.
3.	75-100%	22.7% (17)	28.0% (21)	49.3% (37)	0% (0)	27.
4.	7-100%	28.6% (2)	0% ()	71.4% (5)	0% (0)	29.
Altogether	154-100%	45.5% (70)	13.6% (21)	35.1% (54)	5.8% (9)	
Non-diabetic kidney disease – altogether			48.7% (75)			

type 2 diabetic patients with renal affection, particularly in those without diabetic retinopathy and/or acanthocyturia.

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Ratio of cyclase activating and cyclase inactive parathormone (CAP/CIP) in dialysis patients: correlations with other markers of bone disease

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Abstract

Purpose: **We checked correlation of CAP/CIP with osteoprotegrin (OPG), its soluble ligand (OPGL) and routinely measured parameters of bone turnover in patients treated with peritoneal dialysis (PD) and hemodialysis (HD).**

Material & methods: **In 30 patients (22 HD, 8 PD) we determined serum concentrations of intact parathormone (iPTH), CAP, OPG, OPGL, total Ca, inorganic phosphates (Pi), creatinine, urea, total alkaline phosphatase (AP) and blood pH. CIP was calculated by subtraction of CAP from iPTH. Controls (Cs) included 9 healthy persons in whom iPTH, CAP, OPG and OPGL were measured as well as CIP, CAP/CIP and OPGL/OPG were calculated.**

Results: **Differences between HD and PD patients included dialysis duration, OPGL, OPGL/OPG, AP, Pi, Ca and pH. After adjustment to dialysis duration differences in OPGL/OPG, Pi, Ca and pH remained significant. HD patients differed from Cs in terms of iPTH, CAP, CIP, OPGL, OPG and OPGL/OPG. In whole group of patients iPTH, CAP, CIP but not CAP/CIP correlated negatively with OPGL and OPGL/OPG as well as positively with dialysis duration, OPG and AP.**

Conclusions: **Despite more advanced uremic bone disease in longer dialyzed HD patients than in shorter dialyzed PD ones, CAP/CIP is not different neither between these groups nor Cs persons. CAP/CIP does not seem to be more powerful tool in noninvasive diagnosis of bone disease than iPTH or CAP and CIP alone.**

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Key words: parathormone, CAP/CIP, osteoprotegrin, osteoprotegrin ligand, dialysis.

Introduction

1-84 parathormone (PTH) – cyclase activating PTH (CAP) and 7-84 PTH – cyclase inactive PTH (CIP) are secreted by the parathyroid glands. CAP is a whole PTH molecule, which consists of 84 amino acids, but only the first 34 are essential in keeping of mineral homeostasis. CIP is a C-terminal fragment of 1-84 PTH and has been demonstrated to be an antagonist of CAP with inverse biological activities as it was shown in thyroparathyroidectomized and nephrectomized rats [1]. CAP operates through the type 1 PTH/PTH related peptide (rP) receptor and stimulates synthesis of cyclic adenosinomonophosphoran (cAMP) [2]. Human CIP inhibits bone resorption in vitro via actions independent of the type 1 PTH/PTHrP receptor [3]. CIP appears to operate through a C terminal PTH receptor [4]. CAP is hypercalcemic and increases bone turnover, whereas CIP has been demonstrated to be hypocalcemic and able to lower bone turnover through an inhibition of osteoclast formation and differentiation resulting in an overall inhibition of bone resorption. CIP does not increase synthesis of cAMP [2].

The 2nd generation "intact" PTH (iPTH) assays measure sum of CAP and CIP. Thus, estimation of iPTH, if considered as "active" PTH, provides PTH activity over 30% higher due to detection of CIP additionally to CAP [5]. Such high PTH levels may result in over treatment with D-analogues, leading to adynamic bone disease and soft tissue calcifications. Advanced assay 3rd generation methodology anables to measure biologically active CAP [5,6].

CAP/CIP (the optimal range 1.5-1.8) is recently considered as useful in assessment of bone turnover in dialysis patients [4, 6]. It has been demonstrated to predict bone turnover with a histologically determined 93% predictability. CAP/CIP below unity indicates in dialysis patients adynamic low bone turover (87.5%) [7,8]. More difficult task is to answer the question which value

of CAP/CIP is characteristic for high bone turnover, because among patients with CAP/CIP of 2.0 or higher there are 60% of patients with normal bone turnover [9]. For clinical practice, it is adviced to use value of 1.4 as separating dialysis patients with low bone turnover (CAP/CIP < 1.4) and normal or high bone turnover (CAP/CIP > 1.4) [9].

In this study we checked correlation of CAP/CIP with osteoprotegrin (OPG), its soluble ligand (OPGL) and routinely measured parameters of bone turnover in patients treated with peritoneal dialysis (PD) or hemodialysis (HD).

Material and methods

The study was performed in 30 patients (22 were treated with HD, 8 – with PD). Mean patients' age was 61.6 (36.7 - 79.6) years. Patients were treated with dialysis for 15.2 (1.3 - 186.3) months. Dialysis duration was significantly longer in HD patients than in PD ones (p = 0.014; 26.7, 3.5 - 186.3 months for HD vs 5.0, 1.3 - 26.7 months for PD).

In dialyzed patients serum concentrations of iPTH and CAP were measured by Duo PTH (BioRepair, Sinsheim, Germany). OPG and OPGL were measured by ELISA (Biomedica, Vienna, Austria). Other measured parameters included serum total alkaline phosphatase (AP) activity, serum total calcium (Ca) and inorganic phosphate (Pi) concentrations, blood pH and serum creatinine and urea levels. All the parameters were measured by routinely used methods.

CIP was calculated by subtraction of CAP from iPTH. CAP/CIP and OPGL/OPG were also calculated in examined patients.

Control group (Cs) included 9 healthy persons in whom serum levels of iPTH, CAP, OPG and OPGL were measured as well as CIP, CAP/CIP and OPGL/OPG were calculated.

All results are expressed as median and range. ANOVA for nonparametric data was used to elucidate differences between HD, PD and Cs. Either Student's t-test for non-paired data or Mann-Whitney U test was used to check differences between PD and HD patients. Spearman's and Pearson's coefficients were respectively used to describe correlations between non-normal and normal distributed variables. Results were also adjusted to dialysis duration. The p level less than 0.05 was considered as significant.

Results

There was no statistically significant difference in terms of iPTH, CAP, CIP and CAP/CIP between HD and PD patients. Only HD patients differed from Cs in terms of iPTH, CAP and CIP, but not in CAP/CIP (*Tab. 1*).

Significant differences were shown between HD patients and Cs for OPG (p = 0.004; 7.8, 1.5 - 15.8 pmol/L for HD vs 2.2, 1.0 - 3.9 pmol/L for Cs), OPGL (p = 0.035; 0.6, 0.0 - 10.0 pmol/L for HD vs 3.4, 0.4 - 10.5 pmol/L for Cs) and OPGL/OPG (p = 0.000; 0.10, 0.00 - 1.45 for HD vs 1.23, 0.18 - 5.4 for Cs) as well as between HD patients and PD ones for OPGL (p = 0.016; 2.1, 0.0 - 5.3 pmol/L for PD) and OPGL/OPG (p = 0.003; 0.73, 0.00 - 1.60 for PD). After adjustment to dialysis duration differences in OPGL/OPG between HD patients and PD ones remained significant.

Table 1. **Serum parathormone levels in hemodialysis (HD) patients, peritoneal dialysis (PD) patients and controls**

	HD patients	PD patients	Controls
iPTH (pg/ml)	199.9 (10.3-1266.9)	109.2 (13.7-334.9)	37.4 (18.9-76.8)[a]
CAP (pg/ml)	126.7 (6.5-887.9)	79.7 (9.3-238.6)	23.5 (11.2-42.6)[b]
CIP (pg/ml)	70.4 (3.3-398.9)	48.4 (2.4-129.0)	13.5 (0.9-34.2)[c]
CAP/CIP ratio	1.73 (0.84-4.21)	1.97 (0.90-4.67)	1.45 (0.92-3.61)

No significant changes between HD patients and PD ones
Changes between HD patients and controls:
[a] p = 0.016 [b] p = 0.018 [c] p = 0.019

Significant differences between HD patients and PD ones also included standard parameters related to bone turnover: serum concentrations of Ca (p = 0.004; 2.2, 0.8 - 2.8 mmol/L for HD vs 2.6, 2.2 - 2.6 mmol/L for PD) and Pi (p = 0.003; 2.5, 1.2 - 7.3 mmol/L for HD vs 1.2, 0.99 - 2.1 mmol/L for PD), total AP activity in serum (p = 0.000; 122, 74 - 577 U/L for HD vs 64, 60 - 98 U/L for PD) and blood pH (p = 0.003; 7.31, 7.28 - 7.35 for HD vs 7.43, 7.32 - 7.50 for PD). After adjustment to dialysis duration all these parameters except total AP remained significantly different between both groups of patients.

In whole group of examined patients iPTH, CAP, CIP but not CAP/CIP correlated negatively with OPGL (iPTH: r = -0.377, p = 0.018; CAP: r = -0.356, p = 0.026; CIP: r = -0.383, p = 0.016), OPGL/OPG (iPTH: r = -0.435, p = 0.006; CAP: r = -0.414, p = 0.009; CIP: r = -0.440, p = 0.005) and positively with dialysis duration (iPTH: r = 0.415, p = 0.025; CAP: r = 0.420, p = 0.023; CIP: r = 0.392, p = 0.035), OPG (iPTH: r = 0. 374, p = 0.019; CAP: r = 0.366, p = 0.022; CIP: r = 0.406, p = 0.010) and AP (iPTH: r = 0.606, p = 0.007; CAP: r = 0.577, p = 0.002; CIP: r = 0.617, p = 0.001). iPTH additionally showed correlation with pH (r = -0.514, p = 0.042).

In PD patients correlation of CAP/CIP with serum urea concentration (r = -0.717, p = 0.030) was shown. In HD patients correlation between AP and iPTH (r = 0.619, p = 0.005), CAP (r = 0.605, p = 0.006) and CIP (r = 0.657, p = 0.002) was shown, and between iPTH and serum urea level (r = 0.633, p = 0.036).

Discussion

Comparisons of iPTH, CAP, CIP, CAP/CIP and total AP indicate that secondary hyperparathyroidism is more advanced in longer dialyzed HD patients than in shorter dialyzed PD ones. The later group, if small, may not differ from controls in terms of serum PTH concentrations. CAP/CIP is not able to differentiate HD patients, PD patients, and controls. It suggests

that regulatory system preserving normal proportions between serum CAP and CIP concentrations is not disturbed in dialyzed patients. According to Gao et al. [5], CAP as percent of total PTH is lower and serum CIP concentration is higher in uremic patients. Both these abnormalities acting together or separately may explain a resistance to active PTH in uremia, causing an effect that 3-5 times higher iPTH concentration is needed to keep normal bone turnover [10].

OPG acts as a soluble secreted receptor for OPGL that prevents it from binding to and activating osteoclast differentiation an activation receptor on the osteoclast surface. The biological effects of OPG on bone cells are the opposite of that of OPGL, including inhibition of terminal stages of osteoclast differentiation [11-13], suppression of the activation of mature osteoclasts [12,14], and induction of apoptosis [15]. OPG also inhibits osteoclastic pit formation of mature osteoclasts [12,14] and antagonizes the induction of bone resorption by $1\alpha,25\text{-}(OH)_2D_3$, PGE_2, PTH, IL-1α [12,16], as well as OPGL [16].

Increased OPG level was already shown in HD patients [17, 18]. However, reports on diagnostic value of OPG estimations in serum are controversial. Coen et al. [17] have demonstrated lower OPG concentration in patients with adynamic bone disease than in patients with high bone turnover. Haas et al. [18] reported both OPG and PTH as markers of high turnover osteodystrophy and decreased bone mineralisation in HD patients. We lean towards hypothesis that lower serum OPG level is connected with lower activity of osteoclasts, what appears in adynamic bone disease, and with less compensating production of OPG. When serum PTH increases, OPG also rises to prevent bone destruction associated with PTH action.

CAP/CIP did not show significant correlations with other markers of bone turnover, dialysis duration or blood pH. Such correlations were observed for iPTH, CAP and CIP. It is worthy to notice that in our study in dialyzed patients both CAP and CIP showed a significant positive correlation with AP as it was earlier demonstrated in kidney transplant patients [19]. This may suggest that changes in CAP/CIP can be hardly used to show relations between parameters of bone turnover. Additionally, there were no significant correlations in our study between serum concentrations of total Ca and CAP/CIP. According to the earlier studies of the other authors [4], CAP/CIP decreases in dialysis patients when serum calcium concentration increases and vice versa. In PD patients, lower serum OPG level may contribute to higher serum calcium level because OPG causes a decrease in serum calcium concentration [20].

Our studies indicate that despite more advanced uremic bone disease in longer dialyzed HD patients than in shorter dialyzed PD ones, CAP/CIP is not different neither between these groups nor Cs persons. We conclude that CAP/CIP does not seem to be more powerful tool in noninvasive diagnosis of bone disease than iPTH, CAP and CIP alone, OPGL, OPGL/ /OPG or even AP.

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Serum osteoprotegrin level is lower in peritoneal dialysis patients than in hemodialysis ones

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Abstract

Purpose: Osteoprotegrin (OPG) and OPG-ligand are involved in bone turnover induced by parathyroid hormone (PTH) in renal osteodystrophy. We determined serum OPG level in dialysis patients and checked its correlations with other parameters of bone turnover.

Material and methods: Serum level of OPG, PTH, phosphates (Pi), calcium, total alkaline phosphatase (AP) and pH was determined in 29 peritoneal dialysis (PD) patients and 41 hemodialysis (HD) ones.

Results: OPG level was lower in PD than HD patients (4.0, 2.1-13.4 pmol/L vs 7.9, 0.9-16.5 pmol/L; $p = 0.000$) and in both groups it was higher than in controls (2.2, 1.0-3.9 pmol/L; $p = 0.000$). PD patients had also lower AP (78.0, 34.0-583.0 U/L vs 116.0, 59.0-577.0 U/L; $p = 0.000$) and Pi (1.4 ± 0.4 mmol/L vs 3.4 ± 2.6 mmol/L; $p = 0.000$) than HD patients, but higher calcium level (2.4 ± 0.2 mmol/L vs 2.2 ± 0.3 mmol/L; $p = 0.002$) and pH (7.412 ± 0.051 vs 7.326 ± 0.043; $p = 0.000$). The only correlation displayed in PD patients for OPG was negative one with pH ($r = -0.417$, $p = 0.038$) and in HD patients – positive with Pi ($r = 0.548$, $p = 0.000$). OPG level was elevated in 38 HD (92,7%) and in 15 PD (51,7%) patients. There was correlation between serum OPG and AP ($r = -0.615$, $p = 0.033$) and calcium ($r = 0.575$, $p = 0.040$) in group characterised by normal OPG value. There were no significant correlations in group with elevated OPG level.

Conclusions: Lower serum OPG level in PD patients may be connected with lower activity of osteoclasts and less compensating production of OPG in this group of patients. Lower serum OPG level may contribute to higher serum calcium level in PD patients.

Kay words: osteoprotegrin, parathormone, peritoneal dialysis, hemodialysis.

Introduction

The precise mechanism of different kinds of renal osteodystrophy (ROD) is still not fully understood and ROD is still one of the major complications of chronic renal failure, and is associated with increasing morbidity over time. Recently, it was suggested that osteoprotegrin/osteoptotegrin-ligand (OPG/OPG-L) system is involved in pathogenesis of ROD [1,2].

OPG is a member of the tumor necrosis factor (TNF) – receptor superfamily. Its gene is located on chromosome 8q23-24 [3], and OPG mRNA has wide tissue distribution, that is not restricted to bone or immune tissues [3-5]. OPG is synthesized as a 401 amino acids peptide for the human, of which the signal peptide is cleaved, thus generating the mature peptide (380 amino acids) [3,4]. In contrast to all other TNF-receptor superfamily members, OPG lacks transmembrane and cytoplasmic domeins and is secreted as soluble protein [3-5]. Synthesis of OPG in osteoblastic lineage cells is increased by the cytokines such as interleukin (IL)-1α, IL-1β, TNF-α, TNF-β, 1α,25-$(OH)_2D_3$, bone morphogenetic protein 2 [6] and by antiresorptive agents estrogen [7] and transforming growth factor-β (TGF-β) [8]. Protein level is however decreased by glucocorticoids [9], prostaglandin E_2 (PGE_2) [10] and by the pure estrogen receptor antagonist ICI 182,780 [7]. OPG acts as a soluble secreted receptor for OPG-L that prevents it from binding to an activating osteoclast differentiation and activation

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Figure 1. **Serum osteoprotegrin (OPG) level in dialysed patients and controls**

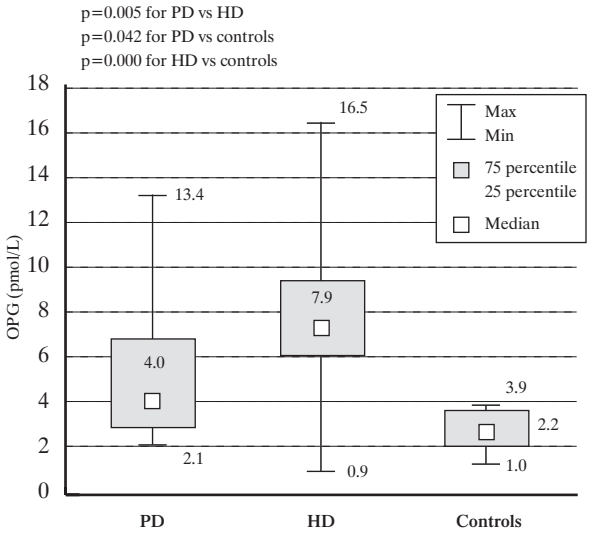
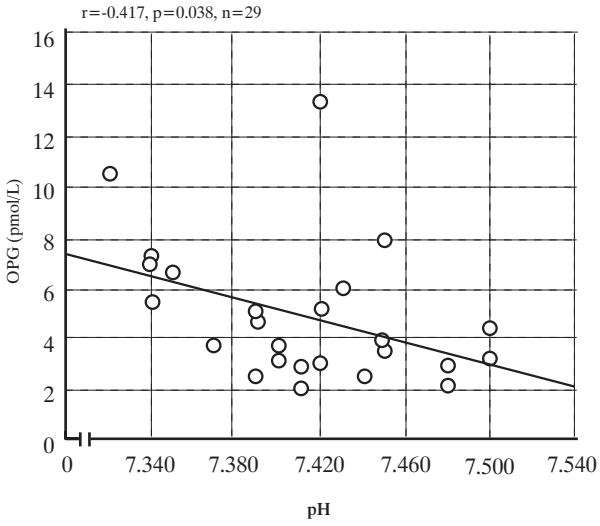


Figure 2. **A correlation between osteoprotegrin (OPG) serum concentration and blood pH in PD patients**



receptor (ODAR) on the osteoclast surface. Thus, the biological effects of OPG on bone cells are the opposite of that of OPG-L, including inhibition of terminal stages of osteoclast differentiation [4,5,11], suppression of the activation of mature osteoclasts [5,12], and induction of apoptosis [13]. OPG also inhibits osteoclastic pit formation of mature osteoclasts [5,12] and antagonizes the induction of bone resorption by $1\alpha,25\text{-}(OH)_2D_3$, PGE_2, parathormone (PTH), IL-1α (5,14), as well as OPG-L [14].

In this study we checked serum OPG concentration in peritoneal dialysis (PD) and hemodialysis (HD) patients and correlated it with routinely measured parameters of bone metabolism.

Material and methods

The study was performed in 29 PD patients (age 55.1 ± 14.5 years, PD duration 11.4; 0.1-57.4 months) and 41 HD ones (age 63.0 ± 10.6 years, HD duration 24.1; 1.1-186.3 months). The causes of end stage renal disease in PD patients were the following: diabetic nephropathy (9 patients), chronic glomerulonephritis (6 patients), tubulointerstitial nephropathy (6 patients), polycystic kidney disease (3 patients), hypertensive nephropathy (2 patients), unknown (3 patients). In HD patients, end stage renal disease was caused by diabetic nephropathy (10 patients), tubulointerstitial nephropathy (9 patients), chronic glomerulonephritis (8 patients), polycystic kidney disease (6 patients), obstructive nephropathy (3 patients), ischaemic nephropathy (1 patients), unknown reasons (4 patients). Difference between HD and PD patients in terms of age and dialysis duration did not achieve statistical significance. The control group (CG) included 13 healthy volunteers.

Serum OPG concentration was measured by enzyme immunoassay (Biomedica, Vienna, Austria) using specific biotinylated OPG detection antibody. In the first step they combine to detected substance and respectively to the precoauted capture anti-OPG antibody and form a sandwich. In the last step OPG is quantitated by an enzyme catalyzed color change detectable on standard ELISA reader. The amount of color developed is directly proportional to the amount of measured substance.

Serum concentration of intact PTH and cyclase activating PTH/cyclase inactive PTH (CAP/CIP) ratio were measured by immunoradiometric assay (DuoPTH, BioRepair, Sinsheim, Germany). Other parameters such as phosphates (Pi), total calcium, total alkaline phosphatase (AP) activity and blood pH were simultaneously measured by routinely used methods.

All results are expressed as mean ± SD or median and range, when appropriate. ANOVA for nonparametric data was used to elucidate differences between HD, PD and CG. Either Student's t-test for paired data or Mann-Whitney U test was used to checked differences between PD and HD patients. Spearman's and Pearson's coefficients were respectively used to describe correlations between non-normal and normal distributed variables. P less than 0.05 was considered as statistically significant.

Results

Serum OPG level was significantly lower in PD than HD patients and in both groups it was higher than in CG (*Fig. 1*). PD patients had also lower AP (78.0, 34.0-583.0 U/L vs 116.0, 59.0-577.0 U/L; p = 0.000) and Pi (1.4 ± 0.4 mmol/L vs 3.4 ± 2.6 mmol/L; p = 0.000) concentrations than HD patients, but higher serum calcium level (2.4 ± 0.2 mmol/L vs 2.2 ± 0.3 mmol/L; p = 0.002) and pH (7.412 ± 0.051 vs 7.326 ± 0.043; p = 0.000). The only correlation displayed in PD patients for OPG was negative correlation with pH (*Fig. 2*) and in HD patients – positive with Pi (*Fig. 3*). Serum OPG level was elevated above normal value in 38 HD (92,7%) and in 15 PD (51,7%) patients. There was significant correlation between serum OPG level and AP (*Fig. 4*) and serum calcium (*Fig. 5*)

Figure 3. **A correlation between serum concentration of osteoprotegrin (OPG) and inorganic phosphates (Pi) in HD patients**

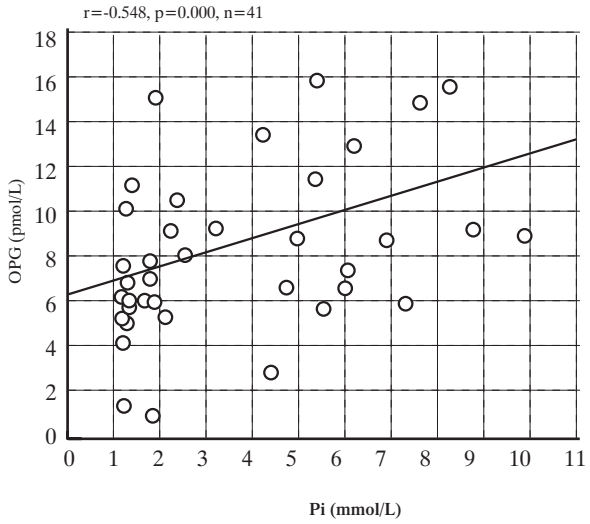


Figure 4. **A correlation between serum osteoprotegrin (OPG) concentration and total alkaline phosphatase (AP) activity in serum of PD patients showing normal serum OPG level**

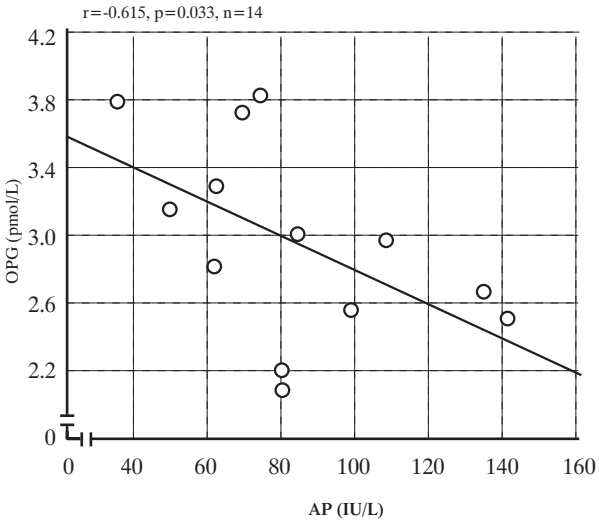
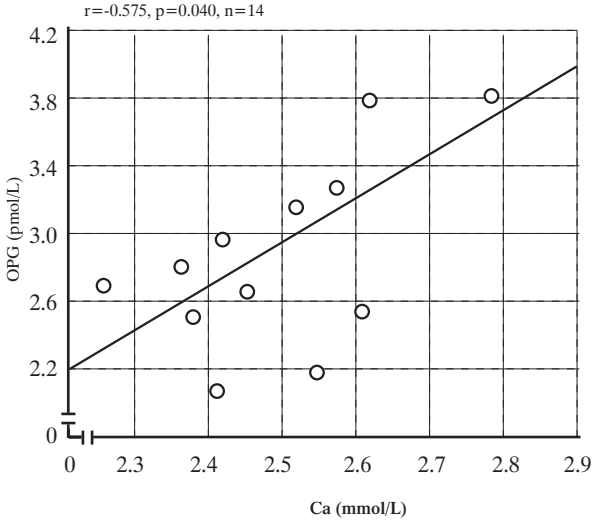


Figure 5. **A correlation between serum concentration of osteoprotegrin (OPG) and total calcium (Ca) in PD patients showing normal serum OPG level**



in group characterised by normal OPG value. There were no significant correlations in group with elevated OPG level.

We did not disclose any significant differences between PD (2.0, 0.9-4.7), HD (1,7, 0.8-4.2) and CG (1.6, 0.9-3.6) in terms of CAP/CIP ratio and it did not correlate significantly with any of examined parameters in PD and HD patients.

Discussion

Our results confirm prior reports on elevated serum OPG in HD patients [1,2]. In our study also PD patients had significantly higher serum OPG concentration than controls, but they had lower level of this cytokine than HD patients. The issue if elevated level of serum OPG is connected with adynamic bone disease (ABD) or high bone turnover disease is not clear [1,2]. We lean towards hypothesis that lower serum OPG level is connected with lower activity of osteoclasts, what appeared in ABD [2], and with less compensating production of OPG. Coen et al. [2] found inverse correlation of serum OPG level with histomorphometric parameters of bone resorption. Such situation may take place in PD patients, who are more predisposed to ABD [15].

Negative correlations between serum OPG level and pH or AP showed in PD patients and positive correlation of OPG with Pi in HD ones seems to confirm relationship between lower activity of osteoclasts and lower serum OPG concentration [2]. Futhermore, lower serum OPG level may contribute to higher serum calcium level in PD patients, because OPG causes a decrease in serum calcium concentration [16].

We did not display correlation between CAP/CIP ratio and level of OPG in serum in any of separated group, however Coen et al. [2] revealed inverse correlation of serum intact PTH and CAP with OPG but only in patients with hyperparathyroidism or mixed osteodystrophy.

In summary, differences in serum OPG concentration showed between dialyzed groups and CG, may indicate that ROD is more advanced in HD patients than PD ones. Lower serum OPG level in PD group is probably connected with lower activity of osteoclasts and less compensating production of OPG. In the about 50% of PD patients osteoclasts function is also disturbed (elevated OPG level). Correlations of OPG with calcium and Pi describe their influence on osteoclasts activity; correlation with pH confirms considerable influence of pH on bone metabolism.

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Protein Z and vitamin K in kidney disease

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Abstract

Purpose: Disturbances in hemostasis are common complications of kidney diseases. Both bleeding diathesis and thromboembolism may complicate the course of chronic uremia. As far as we know, there is a limited data about protein Z in kidney disease.

Material and methods: The aim of our work was to examine plasma protein Z and vitamin K concentrations in nephrotic syndrome (n=34), glomerulonephritis (n=48), kidney transplant recipients (n=80), peritoneally dialyzed patients (n=42) and in the healthy volunteers (n=27).

Results: Vitamin K was significantly lower in nephrotic syndrome when compared to non-nephrotic patients, CAPD and healthy volunteers ($p<0.05$). Protein Z was the highest in CAPD and kidney transplant recipients when compared to any other group. In nephrotic syndrome protein Z was significantly lower when compared to the healthy volunteers, but it did not differ significantly between two groups of patients with chronic renal failure (with and without nephrotic syndrome). Protein Z correlated only with fibrinogen in CAPD, glomerulonephritis and nephrotic patients. Vitamin K correlated with age and albumin in patients with glomerulonephritis, nephrotic syndrome as well as with albumin in CAPD.

Conclusions: Alterations in protein Z might contribute to the enhanced risk of thromboembolic complications in nephrotic syndrome, CAPD and Tx via different and unknown mechanisms. This phenomenon seems to be unrelated to vitamin K status in these patients.

Key words: vitamin K, protein Z, kidney disease.

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Introduction

Hemostasis is a process of blood clot formation at the site of vessel injury. Bleeding or a thrombosis may occur due to missing or dysfunctional moieties of the coagulation or fibrinolytic factors. Abnormal bleeding can result from diminished thrombin generation or enhanced plasmin formation. Conversely, excessive production of thrombin can lead to thrombosis. Renal failure may be associated with a variety of signs and symptoms that are collectively referred to as the uremic state. Both bleeding diathesis and thromboembolism may complicate the course of chronic uremia [1,2]. At present, the incidence of bleeding is apparently declining, whereas thrombotic complications have become the predominant causes of morbidity and mortality in chronic renal failure [2]. Results of both in vitro and in vivo studies suggest that protein Z, a vitamin K-dependent plasma glycoprotein, plays an important role in dampening coagulation [3,4]. Vitamin K is necessary for the posttranslational γ-carboxylation of glutamic acid (Gla), present in several coagulation factors. Gla-mediated Ca^{++} ion binding to the coagulation factors is necessary for their association with phospholipids. This is a critical moment for their hemostatic function. An additional vitamin K-dependent protein, named protein Z, was found to be similar to the coagulation factors VII, IX, X and protein C [3,4]. However, in contrast to them, protein Z is not a serine protesase [3,4]. In the presence of phospholipid vesicles and calcium ions, protein Z serves as a cofactor for the inhibition of activated factor X by protein Z-dependent protease inhibitor. The physiological role of protein Z is still unclear. Controversial data have been published concerning protein Z alterations in different diseases. Low protein Z levels have been associated

with both bleeding tendency [5] and enhanced prothrombotic risk [6]. Recently, a possible role of low protein Z was found to be related to occurrence of acute coronary syndromes [7]. As far as we know, there is a limited data about protein Z in kidney disease. Usalan et al. [8] have reported a high plasma levels of protein Z in hemodialyzed patients. In our preliminary study we found that in nephrotic syndrome vitamin K concentration as well as protein Z were low, but these two findings were unrelated [9]. Since patients with nephrotic syndrome, peritoneally dialyzed and kidney transplant recipients exhibit hypercoagulable state and are particularly prone to thromboembolic complications [10-12], the aim of our work was to examine soluble plasma protein Z and vitamin K concentrations in these populations.

Material and methods

The study was performed on 4 groups of clinically stable patients with renal failure: group I – 48 patients with chronic glomerulonephritis without nephrotic syndrome, group II – 34 patients with nephrotic syndrome, group III – 42 peritoneal dialysis patients maintained on CAPD, group IV – 80 kidney transplant recipients.

In CAPD patients renal failure was due to glomerulonephritis (n=18), chronic interstitial nephritis (n=10), diabetic nephropathy (n=6), polycystic kidney disease (n=5) and other or unknown causes (n=3). All the diabetics were treated with subcutaneous insulin. Blood samples were drawn in the morning when subjects, appeared for routine office assessment of dialysis therapy after an overnight fast. All the CAPD patients were performing four 2 l exchanges. They were using Baxter Twin Bag system or Fresenius Andy Plus system. 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.

80 kidney transplant recipients, with stable graft function (serum creatinine 1.74 ± 0.80 mg/dl) without any 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 agents used in the transplants recipients consisted of cyclosporine (Neoral), azathioprine/mycophenolate mofetil and prednisone. The average dose of cyclosporine was 290 ± 85 mg/day, concentration measured using monoclonal antibodies in the whole blood was 158 ± 62 ng/ml). The average dose of prednisone was 5 mg/day and the highest dose was 10 mg/day. The azathioprine (Imuran) dose was on average 100 mg/day, with a range of doses between 50 and 150 mg/day, the mycophenolate mofetil (CellCept) doses ranged from 1 g/d to 2 g/d. Patients were engrafted for a period of 7 months to 12 years. The underlying renal pathology in patients was chronic glomerulonephritis (n=38), pyelonephritis (n=9), adult polycystic kidney disease (n=8), unknown (n=19), diabetic nephropathy (n=6).

Nephrotic syndrome was due to IgA nephropathy (n=15), focal and segmental glomerulosclerosis (n=11), membrano-proliferative glomerulonephritis in 3 cases, membranous nephropathy in 2 cases, submicroscopic glomerulonephritis in 1 case. Biopsy was not diagnostic in 2 cases. During the study none of the patients have received prednisone, anticoagulants or cytotoxic drugs. Twenty seven sex-and age-matched healthy volunteers served as a control group.

All the patients were informed about the aim of the study and gave their consent. The study was approved by Local Ethical Committee.

The following parameters were assessed: total protein, albumin, cholesterol, triglycerides, urea, total calcium, prothrombin time and activated partial thromboplastin time-APTT by means of standard laboratory methods. Vitamin K concentration was analyzed by HPLC as described previously [13]. Protein Z was assyaed using commercially available kits from Diagnostica Stago, France.

Data expressed as means ± SD and analyzed using Statistica 5.0 computer software. ANOVA or Kruskal-Wallis ANOVA, Pearson or Spearman correlations were used in statistical analysis, when appropriate.

Results

Concentrations of vitamin K were not significantly related to either age or gender in patients on renal replacement therapy (CAPD, Tx), but in GN+NS vitamin K correlated positively with age ($r=0.64$, $p<0.01$). We found that vitamin K concentrations did not differ significantly between all of the groups studied, when all the patients with chronic renal failure were taken together (NS+GN). When they were divided into patients with and without nephrotic syndrome, vitamin K was significantly lower in nephrotic syndrome when compared to non-nephrotic patients ($p<0.05$). In nephrotic patients vitamin K concentration was lower when compared to the patients maintained on CAPD and the healthy volunteers ($p<0.05$). Protein Z was the highest in CAPD patients when compared to any other group. In nephrotic syndrome protein Z was significantly lower when compared to the healthy volunteers, but it did not differ significantly between two groups of patients with chronic renal failure (with and without nephrotic syndrome). Protein Z correlated only with fibrinogen in CAPD patients ($r=-0.78$, $p<0.01$) and NS+GN ($r=-0.73$, $p<0.01$). Vitamin K correlated with albumin in CAPD patients ($r=0.52$, $p<0.05$) and in NS+GN ($r=0.58$, $p<0.05$).

Discussion

Protein Z (PZ) is a 6.2 kDa vitamin K-dependent protein, synthesized in the liver, first described in bovine plasma by Prowse and Esnouf [14]. Later human protein Z, was isolated in 1984 but its physiological function has remained uncertain [15]. It is a 62 kD glycoprotein, highly homologous to factors VII, IX, X, protein C, S and prothrombin. However, protein Z is not a serine protease due to lack of the active centre in its amino acid sequence [3,4]. It has been suggested that protein Z could promote coagulation [16], but since protein Z circulates in complex with protein Z-dependent protease inhibitor (ZPI),

Table 1. **Vitamin K, protein Z and some biochemical markers in patients with nephrotic syndrome (NS), peritoneally dialyzed patients (CAPD), kidney transplant recipients (Tx), patients with chronic glomerulonephritis (GN) and healthy volunteers (CG)**

	NS n=34	CAPD n=42	Tx n=80	GN n=48	CG n=27
age (years)	52±19	49±17	48±12	55±18	45±16
BMI (kg/m^2)	25.4±4.2	23.9±3.4	25.2±297	23.5±4.1	24.9±4.8
cholesterol (mg/dl)	238±58^{o}	223±47**	219±41**	209±65**	178±38
triglycerides (mg/dl)	254±69*	159±72***	201±44***	127±52**	93±29
total protein (g/l)	6.19±0.52*	6.22±0.69**	6.89±0.67	6.48±0.71**	6.95±0.61
albumin (g/l)	3.49 ±0.50*^{oo}	3.51±0.54**^{oo}	4.26±0.44	3.73±0.42**^{oo}	4.35±0.56
urea (mg/dl)	85.3±28.8***	127.5±41.2***	99.4±37.1***	85.7±42.3***	29.1±5.5
Vitamin K (ng/L)	93.8±17.5*^{o}	108.9±20.9$^{\&}$	109.4±21.5	108.8±17.2#	109.5±13.1
Protein Z (ng/ml)	0.48±0.27**$^{\&\&\&}$	1.58±0.44*^{o}	0.83±0.35*$^{\&\&}$	1.05±0.27$^{\&}$	1.19±0.32$^{\&}$
prothrombin time (secs)	12.5±0.7	12.4±0.9	13.0±1.0	13.2±0.5	12.3±1.0
aptt (secs)	30.8±7.9	34.5±7.8	37.0±8.1	36.2±10.1	36.4±8.2
total Ca (mmol/l)	2.14±0.31^{oo}##	2.09±0.31^{oo}##	2.29±0.33	2.29±0.23	ND

Values given are means ± SD,
* p<0.05, ** p<0.01, *** p<0.001 vs control group
#p<0.05 vs NS
$^{\&}$ p<0.05, $^{\&\&}$ p<0.01, $^{\&\&\&}$ p<0.001 vs CAPD
^{o}p<0.05, ^{oo}p<0.01, vs Tx
ND – not done

this limits the action of protein Z to a cofactor for the inhibition of activated factor X [17]. In the last few years, limited and controversial data were published on the changes of protein Z in various diseases. Very low plasma levels of protein Z were observed under oral anticoagulant treatment [18]. The cause of this phenomenon might be increased protein Z binding on the surface of endothelial cells or its consumption during coagulopathy. In our study we chose population of kidney patients prone to hypercoagulability. Nephrotic syndrome is known to carry an increased risk of thrombosis by a number of mechanisms. Continuous ambulatory peritoneal dialysis (CAPD) with its tendency to hypoalbuminemia and hypertriglyceridemia as well as transperitoneal protein loss appears to mimic the metabolic abnormalities that account for the hypercoagulability in the nephrotic syndrome [19]. Similarly stable kidney transplant recipients manifest a chronic hypercoagulable state with an increased risk of thrombotic complications, which also appears to be multifactorial. In our study we found that in patients with nephrotic syndrome, protein Z concentration was significantly lower than in the healthy volunteers and in CAPD and kidney transplant recipients it was significantly higher than in the control group. Significant protein losses through the peritoneum may be counterbalanced by the possible increase in protein synthesis in CAPD as a result of a nonspecific stimulation of the liver to protein production due to a lowered oncotic pressure. Thus, protein Z synthesis in CAPD patients may be increased via this mechanism. In nephrotic syndrome urinary protein loss is usually lesser than in transperitoneal protein loss in CAPD patients. On the other hand, preliminary reports suggest that protein Z could be a negative acute phase reactant [20]. In fact, inflammation (very often subclinical with CRP within normal ranges) is a frequent finding in dialyzed patients as a one of the components of MIA (malnutrition, inflammation, atherosclerosis) syndrome. In our study we observed a negative correlation between protein Z and fibrinogen in nephrotic syndrome and CAPD. Similarly, Fedi et al. [7] observed a lower protein Z levels in patiens with acute coronary syndromes and fibrinogen levels over 400 mg/dL. In the only one paper regarding protein Z levels in dialyzed patients, Usalan et al. [8] compared plasma protein Z levels in 10 hemodialysis patients (6 M, 4 F, mean age 36±11) and 10 healthy normal controls (5 M, 5 F, mean age 34±8). They found increased mean plasma protein Z levels in haemodialysis patients over healthy controls. They suggested that increased level of protein Z, which influenced the action of thrombin on its protein substrates and inhibitors might contribute to the haemostatis alterations in end-stage renal failure patients, in addition to other well known abnormalities in the coagulation and fibrinolytic system. In contrast to our study, the number of patients was relatively small and they were significantly younger.

It is known that vitamin K is needed for the carboxylation of osteocalcin, matrix Gla protein in bone, prothrombin, protein S and protein Z [21]. However, the specific function of these proteins is still unknown. Therefore, we tried to find correlations between vitamin K status and protein Z. In none of the group of patients studied vitamin K was related to protein Z levels.

Conclusions

Our results add further weight to the possible role of protein Z in the hemostatic disturbances in kidney diseases. The most interesting finding of our study is the observation

that in nephrotic syndrome vitamin K concentration is low as well as protein Z. Protein Z alterations might contribute to the enhanced risk of thromboembolic complications in nephrotic syndrome, CAPD and stable kidney transplant recipients, however, these findings seems to be unrelated to vitamin K status in these patients.

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The expression of two alternative transcription forms of platelet-derived growth factor-A chain in the normal human kidney and in glomerulonephritis

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Abstract

Purpose: Up to now, a role of platelet-derived growth factor (PDGF)-AA in glomerulonephritis (GN) remains unclear. PDGF-A chain may be produced in two forms, as a result of the alternative splicing.

Material and methods: We examined the expression of this growth factor in the renal tissue of 57 patients with GN and seven normal kidneys (NK). The gene expression of PDGF-A was examined by reverse transcriptase-polymerase chain reaction. Sets of primers allowing distinction between the two forms of transcripts were used. Specificity of the PCR products was confirmed by restriction enzyme analysis and sequencing. The expression of PDGF-AA/AB was also evaluated by immunohistochemistry.

Results: Compared to NK, the expression of PDGF-A gene was higher in the renal tissue with GN. This expression was higher in non-proliferative GN (NPGN) than in proliferative forms of GN (PGN) (1.24±0.34 vs. 0.86±0.14). In NK, both forms of transcripts (N=4) or only the short one (N=3) were found. In 45.5% of patients with NPGN, only the short form could be detected. In contrast, in 68.6% of patients with PGN both or only the longer form of transcripts were found. In NK, a faint staining for PDGF--AA/AB was observed within glomerular capillaries, whereas a statistically significant increase in this protein expression was particularly stated in NPGN.

Conclusions: These results suggest that the production of the longer PDGF-A chain variant is associated with glomerular cells' proliferation. However, the higher expression of PDGF-AA/AB protein in NPGN could indicate an essential role of this growth factor in the maintaining the glomerular architecture.

Key words: platelet-derived growth factor-A chain, alternative splicing, glomerulonephritis, endothelial cells, maintaining the glomerular architecture.

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Introduction

Platelet-derived growth factor-A (PDGF-A) is believed to be a mediator of smooth muscle cell hyperplasia in hypertension and atherosclerosis [1-4]. Increased expression of this growth factor has also been shown in arteries of rejected renal grafts [5-6]. Recently, PDGF-A involvement in the development of interstitial fibrosis in the renal tissue with diabetic nephropathy has been suggested [7]. Though, the results regarding the expression and a role of PDGF-A in the evolution of glomerulonephritis (GN) remain unclear up to now. In previous studies, we demonstrated the expression of PDGF-A mRNA in IgA nephropathy (IgAN) by the reverse transcriptase-polymerase chain reaction (RT-PCR) [8]. Later, Terada et al. [9], using a similar method, have confirmed the expression of mRNA for this growth factor in various forms of GN. In this study, the expression of PDGF-A gene in IgAN did not differ from that found in the non-proliferative forms of GN (NPGN). However, the results concerning the expression of PDGF-A protein in different morphological forms of GN are conflicting so far [10-11]. In the study of Taniguchi et al. [10], performed on the renal tissue with features of IgAN, the expression of PDGF-AA has been localized to the mesangial areas. In IgAN, mesangial staining for this growth factor has also been observed by Stein-Oakley et al. [11]. Since only a faint glomerular staining for PDGF-AA has been found in focal-segmental glomerulosclerosis (FSGS) in the latter study, it has been suggested that PDGF-AA might

Table 1. **Histological and clinical data of patients at the time of renal biopsy**

Morphology	No of patients	Sex (F/M)	Age (y) Mean/Range	Serum Creatinine μmol/L (Mean ± SEM)	Proteinuria (g/24 h)
Non-proliferative GN	22	13/9	36 (11-71)	91.9 ± 76.9	5.0 ± 1.1
IMGN	14	7/7	45 (11-71)	117.5 ± 55.7	5.5 ± 1.4
FSGS	8	6/2	18 (13-22)	64.5 ± 11.5	3.2 ± 0.3
Proliferative GN	35	19/16	33 (9-64)	83.2 ± 7.1	3.9 ± 1.0
MesPGN	32	18/14	32 (9-64)	78.6 ± 11.5	4.3 ± 1.1
MPGN	3	1/2	31 (16-60)	97.3 ± 9.7	1.2 ± 0.2

NOTE: Values expressed as mean (range); mean ± SEM, or number of patients.
Abbreviations: IMGN, idiopathic membranous GN; FSGS, focal-segmental glomerulosclerosis; MesPGN, mesangial proliferative GN, MPGN, membranoproliferative GN

be involved in the proliferative response of mesangial cells in IgAN [11]. However, Alpers et al. [12] have earlier shown that podocytes of the mature human kidney express the PDGF-AA protein. Recently, Yang et al. have not only confirmed this finding in the normal human kidney, but also demonstrated a significant increase in the podocyte expression of PDGF-AA in the Denys-Drash syndrome [13].

The gene encoding PDGF-A chain is located on the 7p22 position of chromosome 7, and spans 7 exons [14-15]. PDGF-A chain primary transcripts may be spliced into the two different mRNAs, omitting or including the 69 base pairs' (bp) long sequence of exon 6 [15-16]. The sequence of exon 6 has been shown to be responsible for coding the retention motif [17-18]. Transcription of the long form of PDGF-A chain has been suggested to contribute to smooth muscle cells' hyperplasia in atherosclerotic lesions [20]. This is thought to be a consequence of an increased binding of PDGF-AA dimers containing the sequence encoded by the exon 6 to the cell surface- and matrix-associated glycosaminoglycans [19].

The aim of our study was to examine the gene and protein expression of PDGF-A in the normal kidney and in different morphological forms of GN. Patients with proliferative (PGN) and non-proliferative forms of GN (NPGN) were included into the study. Our study revealed the existence of two differentially spliced forms of PDGF-A chain in the human renal tissue. Moreover, the long form of PDGF-A transcript was more often detected in PGN than in NPGN. In addition, glomerular endothelial cells were revealed to express the PDGF-AA/AB protein in the normal kidney. An essential increase in the expression of this protein was observed in GN, particularly in the absence and/or in the association with mild glomerular cells' proliferation.

Material and methods

Patients

Fifty-seven patients with biopsy-proven GN were included into the study: 32 with mesangial proliferative GN (MesPGN), 3 with membranoproliferative GN (MPGN), 14 with idiopathic membranous GN (IMGN) and 8 with FSGS. The diagnosis was based on morphological and immunopathological examinations of renal biopsy specimens. Histological and clinical data of patients are summarized in *Tab. 1*.

Renal biopsies

Renal biopsy material remaining after the immunopathological diagnosis was used in this study. Informed consent was obtained from all the patients. Tissue preparations for immunohistochemistry and RNA isolation were performed as described elsewhere [8]. The RT-PCR analysis of PDGF-A chain gene expression was performed on fragments from the biopsy tissue of all the patients. All these fragments were also checked for the existence of two alternatively spliced PDGF-A chain transcripts. The amounts of tissue remaining after the diagnosis were sufficient for immunohistochemical evaluation of PDGF-AA/AB protein in 17 patients: 11 with MesPGN, 1 with MPGN, 3 with IMGN, and in 2 with FSGS. Normal-appearing kidney tissue from patients undergoing tumor nephrectomy (N = 7) served as controls for both methods used.

RNA isolation and RT-PCR

Total RNA was extracted from renal biopsy specimens and subsequently reverse-transcribed into cDNA as previously described [21]. Sets of primers with lengths of the amplified PCR products and annealing temperatures for particular pairs of primers are listed in *Tab. 2*. For semiquantitative analysis of PDGF-A gene expression, primers' pair 1 was selected, which amplified the sequence spanning exons 1 through 4. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The sequences of 5' and 3' primers for GAPDH were GAGTCAACGGATTTGGTCGT and GTTGTCATGGATGACCTTGG [22], respectively. For the analysis of both short and long forms of PDGF-A chain transcripts, primers' pairs 2 and 3 were selected, which amplified the sequence spanning exons 3 through 7 (see *Tab. 2*). The reaction mixture was subjected to 40 cycles of PCR amplification. The amplification profile consisted of an initial denaturation step at 96°C for 5 min, followed by denaturation at 94° for 1 minute, annealing at the appropriate temperature (*Tab. 2*), and extension at 72°C for 1 minute and 30 seconds. The PCR products were electrophoretically separated on 2% agarose gel (Serva Electrophoresis, Heidelberg, Germany) and visualized by ethidium bromide staining.

Table 2. **Sets of primers and PCR conditions used in the study**

No	Forward 5'-3'	Reverse 3'-5'	Size (bp)	Anneal. temp. (°C)
1	ATGAGGACCTTGGCTTGC Exon 1 (839-856) [23]	GGGACAGCTTCCTCGATGCT Exon 3/4 (1097-1117) [9]	278	56
2	AGCATCGAGGAAGCTGTCCC Exon 3/4 (1097-1017) [9]	TTCTCCCGAGTGTTCTCGGAC Exon 7 (1607-1628) [24]	531 462	62
3	CTCCCGCGTCCACCACCGCAGCGTC Exon 4 (1265-1278) [25]	TTCTCCCGAGTGTTCTCGGAC Exon 7 (1607-1628) [24]	363 294	56

Location for primers is based on the published NCBI sequence (accession no NM_002607)

For PCR products obtained with primers' pair 1 and for the short form of transcript, original cDNA for the short variant of PDGF-A chain in the plasmid puC13 (kindly supplied by Dr. Ch. Betsholtz, University of Uppsala, Sweden) [14] linearized with SmaI was used as a positive control. Negative controls consisted of buffer alone and/or non-reverse-transcribed sample RNA.

Analysis of PCR products

To confirm the specificity of PCR products obtained with the pair 2 of primers, restriction enzyme analysis was performed. The PCR products were purified using the Clean-Up kit for DNA purification (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's instructions. The kit is based on the of DNA ability to absorb to silica-coated surfaces in the presence of chaotropic salts. After purification, the PCR products were resuspended in 25 μL of diethyl pyrocarbonate (DEPC) treated water (Fermentas, Vilnius, Lithuania) and subjected to digestion by the MboI restriction endonuclease (Fermentas). Briefly, 25 μl of purified PCR product was added into the reaction mixture containing MboI specific restriction buffer, 2 U of MboI and the DEPC treated water to the final volume of 30 μL. Overnight incubation at 37°C was performed. After digestion, products were separated on 8% non-denaturing polyacrylamide gel (Serva) and silver stained according to the method of Budowle et al. [26].

For the PCR products obtained with the third set of primers, cycle sequencing of the long and short forms of transcripts (in both directions) was performed by the means of fmol® DNA Cycle Sequencing System (Promega Biotech). Briefly, 5-10 fmol of the purified PCR product was dissolved in the mixture containing 5× sequencing buffer (250 mM Tris-HCl, pH 9.0 at 25°C; 10 mM $MgCl_2$), 1.5 pmol of CY5 (indodicarbocyanine 5-1-O- (2--cyanoethyl)-(N, N-diisopropyl)-phosphoramidite)-end-labeled primer, and 5U of Sequencing Grade *Taq* DNA Polymerase, and DEPC water to the final volume of 16 μL. Then, the mixture was separated into 4 tubes, 4 μL to each, containing 2 μL of the deoxyribonucleoside triphosphates (dNTP) supplemented with a limiting amount of a different dideoxyribonucleoside triphosphate (ddNTP). The amplification profile consisted of an initial denaturation at 95°C for 2 minutes, followed by denaturation at 95°C for 30 seconds, annealing at 42°C for 30 seconds and extension at 70°C for 1 minute. Products were then separated on the Repro Gel™ Long Read (Amersham Biosciences, Uppsala, Sweden) in 0.5 × TBE buffer (890 mM Tris-borate, 890 mM boric acid, 20 mM EDTA) at 55°C for 750 minutes using an automated sequencer Alf® Express II (Amersham Biosciences). Data obtained by sequencing were analyzed by the means of Alf Win™ Sequence Analyser 2.10 software (Amersham Biosciences) and compared to that published in the NCBI sequence database.

Immunohistochemistry

The immunostaining procedure was performed on acetone-fixed cryostat sections using the alkaline phosphatase – anti-alkaline phosphatase method as previously described [8,21]. A rabbit anti-human polyclonal antibody against PDGF-AA (Chemicon International, Temecula, USA) was applied in this study. An antibody to CD31 (Dako A/S, Glostrup, Denmark) was used as a marker of endothelial cells. Control experiments were conducted by omitting the incubation with the primary antibody, as well as with substitution of the primary antibody with non-immune murine serum.

Statistical analysis

Results of RT-PCR and immunohistochemical evaluation of PDGF-A expression were analyzed in particular groups of patients according to their morphological classification. Images of ethidium bromide-stained bands for PDGF-A and GAPDH cDNAs were photographed using a system with camera supported with a darkroom (Vilbert Loumart, Torcy, France). The intensity of the bands was densitometrically measured with the BioGENE computer software (Vilbert Loumart). All PDGF-A signals were normalized to mRNA levels of GAPDH and expressed as a ratio.

Mean glomerular expression scores were calculated for the examined protein, based on the staining intensity (I) and the percentage of glomerular stained area (A) by applied antibody according to the formula: mean glomerular expression score $= I\ (1) \times A\ (1)/100\% + I\ (N) \times A\ (N)/100\%/N$, where N was the number of glomeruli in the renal specimen. Staining intensity was graded from 0 to 3 points according to the following scale: zero – no immunoreactivity, 1-weak immunoreactivity, 2-moderate intense immunoreactivity, and 3-dense marked immunoreactivity.

All values in the text and figures are presented as mean ± SEM. Non-paired Mann-Whitney test was used to test differences between particular groups of patients. The level of significance was set at $P<0.05$.

Figure 1. Examples of RT-PCR evaluation of PDGF-A expression (band at 278 bp) in comparison to GAPDH expression (band at 482 bp) in kidney samples: Lanes 1, 2 and 3, normal kidneys, lanes 4 and 5, MesPGN, lanes 6 and 7, IMGN, lane 8, negative control (buffer)

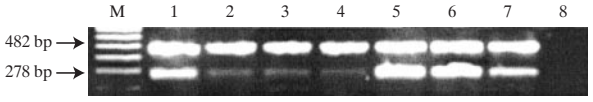


Figure 2. Results of RT-PCR evaluation of PDGF-A chain expression in normal kidneys (NK), proliferative glomerulonephritis (P), and non-proliferative glomerulonephritis (NP)

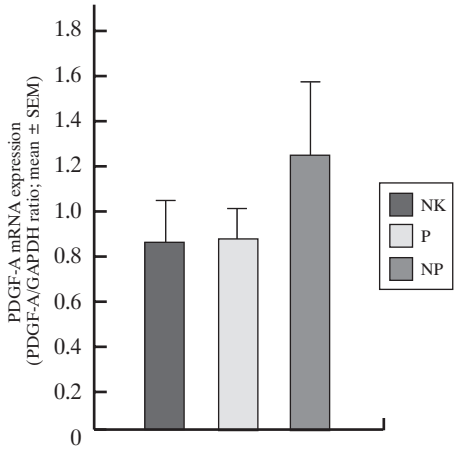


Figure 3. Examples of two differentially spliced variants of PDGF-A chain transcripts in controls and diseased kidneys: lane 1, original PDGF-A chain cDNA without the sequence of exon 6 (positive control); lanes 2 and 4, normal kidneys; lanes 5 and 7, MPGN; lane 6, IMGN, lanes 8, 9, and 12, MesPGN; lanes 10 and 11, FSGS; lane 13, negative control (non-reverse-transcribed RNA)

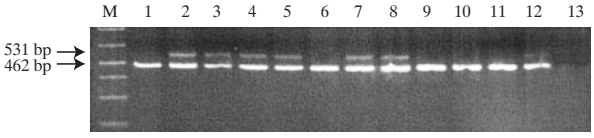
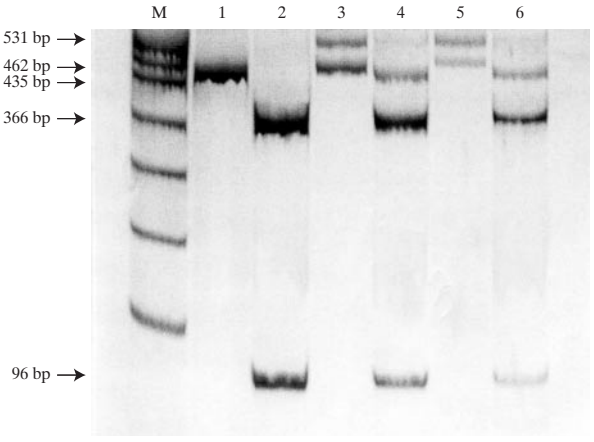


Figure 4. Results of the restriction analysis with MboI of both variants of PDGF-A chain transcripts. The products were separated electrophoretically on 8% polyacrylamide gel and silver stained. Lane 1, short form of PDGF-A chain transcript before digestion (band at 462 bp); lane 2, short form of PDGF-A transcript after digestion with MboI (bands at 366 and 96 bp); lanes 3 and 5, both forms of PDGF-A transcript before digestion (bands at 531 and 462 bp); lanes 4 and 6, both forms of PDGF-A transcript after digestion with MboI (bands at 435, 366 and 96 bp)



Results

Evaluation of PDGF-A gene expression and analysis of transcript variants

By RT-PCR, the PDGF-A chain gene expression was stated in both the normal and diseased kidneys (*Fig. 1*). Fragments of the same length were amplified from the original PDGF-A chain cDNA and reverse transcribed cDNAs from biopsy tissue. In addition, cycle sequencing analysis confirmed that the sequence of the obtained PCR product was identical to that deposited in the NCBI database (not shown). Compared to normal kidneys, higher levels of PDGF-A mRNA were observed in GN (0.84±0.19 versus 1.0±0.16) (not shown). The expression of PDGF-A mRNA in NPGN exceeded that in PGN, although the difference between these groups did not reach statistical significance (*Fig. 2*).

Using the primers' pair 2, PCR products of 462 and 531 bp were observed both in the normal kidney (NK) and in the renal tissue with GN (*Fig. 3*). With respect to the shorter form of detected transcripts, fragment of the same length was amplified from the original PDGF-A chain cDNA. Restriction enzyme analysis of these products with MboI resulted in bands of the expected length, 366 and 96 bp for the short form of PDGF-A chain transcript, and 435, 366 and 96 bp when two forms of transcripts were detected (*Fig. 4*).

Both forms of PCR products obtained with the third set of primers were subjected to the sequencing analysis. They disclosed the 100% similarity with the sequences deposited in the NCBI database (*Fig. 5* and *Fig. 6*).

In four out of seven NK examined, both the long and short forms of PDGF-A transcripts were detected, whereas in the remaining three cases only the short form could be observed. Out of 57 patients, both forms of transcripts were found in 31. In 23 of them, only the short form of PDGF-A chain mRNA was present, whereas the long form singly was detected in 3 patients. Interestingly, the expression of PDGF-A mRNA was significantly higher in the group with the only short variant of transcripts detected (*Fig. 7*). Among these patients were all individuals with FSGS, 4 with IMGN and 11 with MesPGN. The short form of PDGF-A chain mRNA was less frequently detected in PGN. In 68.6% of them, both or only the longer form of transcripts were found.

Figure 5. **Sequencing analysis of the long splice variant of PDGF-A chain. An arrow indicates the boundary between the exons 5 and 6**

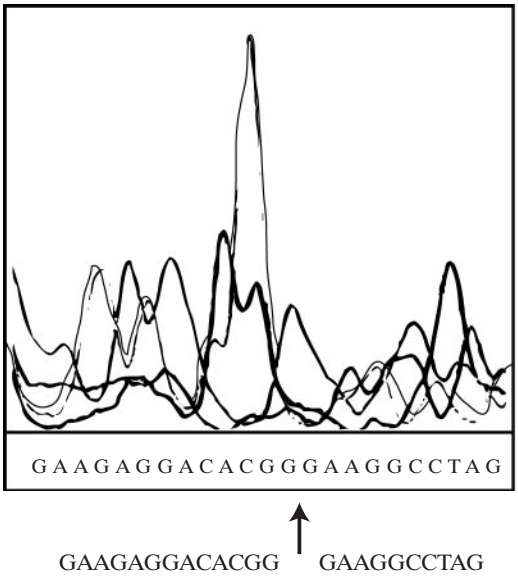


Figure 6. **Sequencing analysis of the short splice variant of PDGF-A. An arrow indicates the boundary between the exons 5 and 7**

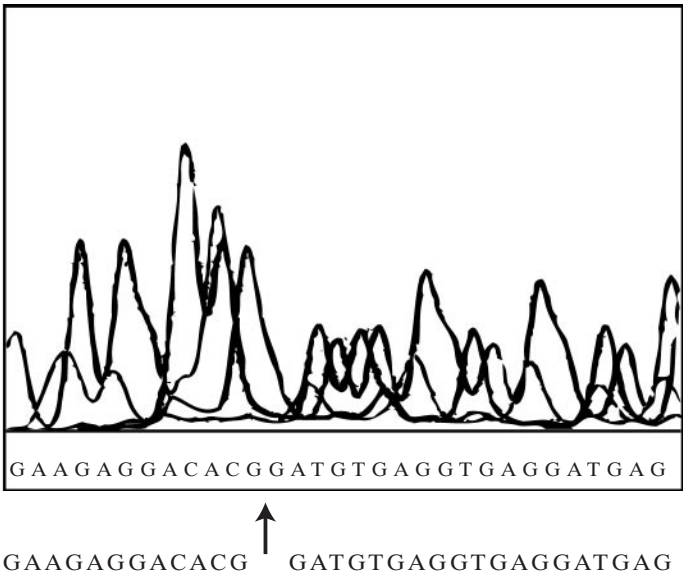
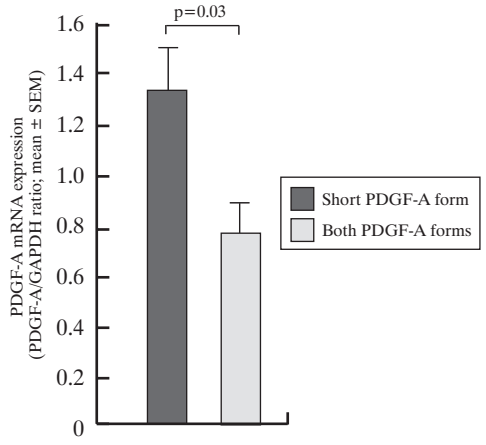


Figure 7. **RT-PCR evaluation of PDGF-A chain expression in patients presenting with only the short splice variant of PDGF-A chain and both splice variants of transcripts**



Evaluation of PDGF-AA/AB protein expression

A faint glomerular immunoreactivity for PDGF-AA/AB was observed in NK (*Fig. 8A*). In the tubulointerstitial compartment, tubular epithelial cells and endothelial cells in the interstitial vessels were positive for PDGF-AA/AB. In GN, the glomerular expression of this protein varied greatly with a staining pattern resembling that for CD31 (not shown). In PGN, the expression of PDGF-AA/AB protein depended on the degree of mesangial cells' proliferative response. Marked immunoreactivity for this growth factor was observed in glomeruli with no or mild mesangial cells' proliferation (*Fig. 8B*), whereas only a faint reaction was noticed in advanced lesions in the course of MPGN (*Fig. 8D*). In NPGN, the glomerular expression of PDGF-AA/AB was increased in early stages of IMGN or FSGS and significantly reduced in the presence of advanced sclerotic alterations (*Fig. 8C*).

Semiquantitative evaluation of the glomerular expression of PDGF-AA/AB in NPGN and PGN, compared to NK, is shown in *Fig. 9*.

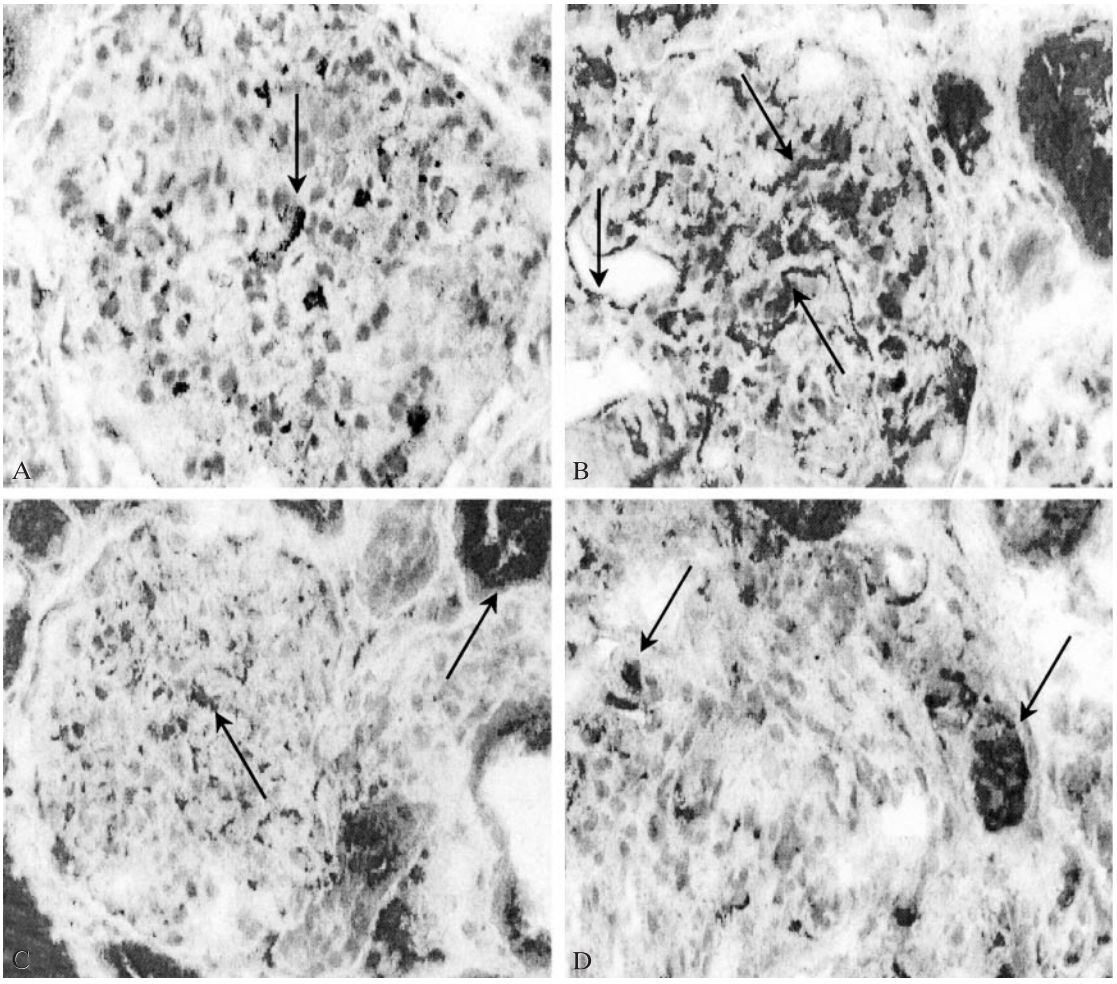
Clinical-pathological correlation

No relationship could be stated between the expression of PDGF-A mRNA or protein and serum creatinine levels or degree of proteinuria in different morphological forms of GN. Intriguingly, a higher expression of PDGF-A mRNA and protein was observed in NPGN. As stated above, the expression of the shorter PDGF-A transcript was more often detected in this group of patients.

Discussion

Our RT-PCR study showed that the gene expression of PDGF-A chain in renal specimens with GN exceeded that in the normal kidney. Previously, only Terada et al. [9] have used a similar method to evaluate the expression of PDGF-A chain mRNA in the renal tissue with GN. Though, the expression of PDGF-A chain mRNA in the normal kidney has not been examined at all in this study. The authors compared the gene expression of PDGF-A chain in IgAN, IMGN, FSGS, and minimal change disease. They stated that the expression of this growth factor was higher in IgAN, but it did not differ significantly from that found in the other types of GN grouped together [9]. In contrast, in our study, a higher expression of PDGF-A chain mRNA was observed in the renal tissue of patients with NPGN than in PGN, although the difference between these groups did not reach statistical significance. One of the reasons for the lack

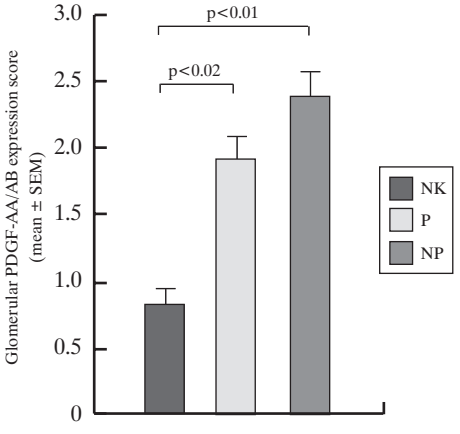
Figure 8. (*A*) Immunoreactivity for PDGF-AA in a normal glomerulus. Some endothelial cells are positive. (*B*) Increased immunoreactivity for PDGF-AA in a glomerulus from a patient with IgA nephropathy and mild mesangial cell proliferation. (*C*) A glomerulus from a patient with an advanced stage of FSGS. The reactivity for PDGF-AA is comparable to that in the normal kidney. (*D*) PDGF-AA immunoreactivity in a glomerulus with features of membranoproliferative GN is even lower than that observed in the normal kidney



of significance might have been the fact that we used the total RNA isolated from the whole remaining biopsy fragment for PDGF-A chain evaluation. However, although Terada et al. performed their study on the RNA isolated only from glomeruli, they amplified the PDGF-A chain sequence spanning exons 3 through 6, and thus they were able to detect only the long transcript [9].

To our knowledge, our study is the first one, which analyzed the expression of two alternative forms of PDGF-A chain transcripts in the normal kidney and in different morphological forms of GN. Digestion with a restriction enzyme and, more notably, sequencing analysis confirmed the specificity of the obtained PCR products. We observed significantly higher levels of mRNA for PDGF-A chain in cases with the only short form of transcripts detected. Intriguingly, in this group were predominantly patients with NPGN, in particular all the patients with FSGS. In contrast, the long form of PDGF-A chain transcript was more often found in PGN. In the latter respect, also in the study of Terada et al. [9] transcripts for the longer form of PDGF-A chain were more frequently detected in patients

Figure 9. Evaluation of PDGF-AA glomerular expression in normal kidneys (NK), proliferative glomerulonephritis (P) and non-proliferative glomerulonephritis (NP)



with IgAN, i.e. the proliferative form of GN, than in those with NPGN. Thus, both studies would suggest that transcription of longer PDGF-A chain form is associated with glomerular cells' proliferation.

In vitro, both forms of PDGF-A chain transcripts have been found in the human smooth muscle arterial cells (VSMC), adult vein endothelial cells (VEC) and monocyte-derived macrophages (M) [19]. In VSMC, the short PDGF-A chain transcript was 8 times more abundant than that of the long form. The long and short PDGF-A chain mRNAs were expressed at similar levels in monocyte-derived macrophages. In contrast, a constant and comparatively high production of the long PDGF-A chain transcript, representing approximately 44% of the total PDGF-A mRNA was observed in VEC [20].

In the latter context, results of our immunohistochemical studies point to the glomerular EC as to the most likely source of PDGF-AA protein in glomeruli. In NK, only trace amounts of this protein could be detected within the glomerular capillaries. An essential increase in the immunoreactivity for PDGF-AA was observed in GN. Furthermore, a significantly higher expression of this growth factor was found in NPGN than in PGN. These results are in disagreement with that obtained by others [10-13]. However, inconsistent are also the results of the above-cited studies. Two different antibodies against PDGF-AA (both polyclonal) were used in these studies. One of them stained mesangial areas in IgAN and gave negative results in NK [10-11], whereas the other stained podocytes both in NK and in the disease condition [12-13]. We used an antibody that in addition to other applications is also recommended for neutralization of the biological activity of human PDGF-AA. Moreover, opposing to all the above studies that were carried out on deparaffinized sections, our study was performed on the acetone-fixed tissue. In the light of these data, both cross-linking effects of formalin on proteins and specificity of the antibodies used to detect the PDGF-AA protein could have impact on the obtained results. Further, one has to realize that an anti-PDGF-AA antibody is able to detect also the PDGF-AB dimer.

Nevertheless, given the specificity of the antibody used, increased expression of PDGF-AA observed in our patients with NPGN appears to be a confusing finding. In vivo, the expression of PDGF-AA has been demonstrated in vascular EC of arteries undergoing acute rejection. The authors suggested that PDGF-AA chain expression might be a marker of EC injury or activation in this condition [5-6]. Recent in vitro results propose a new role for PDGF-AA in vascular remodeling. It has been shown that PDGF-AA and PDGF-BB are released from activated arterial EC. But, PDGF-AA inhibits PDGF-BB-mediated VSMC migration and this is regulated at the receptors level. Namely, PDGF-AA induces the expression of PDGF-α receptor (PDGF-αR) in VSMC [27]. This receptor activates JNK-1 signaling and thus counteracts the activity of PDGF-β receptor (PDGF-βR), i.e. the signaling receptor of PDGF-BB [28].

Increased expression of PDGF-BB and PDGF- R with the mitogenic consequences of this axis has long been implicated in the pathogenesis of GN [8-11,29]. On the other hand, the increased expression of PDGF-αR has been shown in vitro and in vivo on mesangial cells [11,30]. Taking into account the new data on PDGF-AA action, increased expression of this growth factor in GN would indicate its protective role aimed at inhibition of mesangial cell proliferation and maintaining the glomerular architecture.

In conclusion, our study showed the existence of two alternatively spliced variants of PDGF-A chain mRNA in the normal human kidney and in GN. The short form of PDGF-A chain was more frequently detected in NPGN and accompanied by a higher expression of PDGF-AA protein. However, further studies including parallel examination of PDGF-αR and PDGF-BB are necessary to signify the actual role of PDGF-AA in GN.

Acknowledgments

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Soluble adhesion molecules in children and young adults with chronic renal failure treated conservatively

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Abstract

Purpose: Chronic renal failure (CRF) patients present with signs of immunodeficiency, such as increased incidence of infections. Cell adhesion molecules, determining leukocyte migration, may be responsible for the impaired immune response. The aim of the study was to measure soluble (s) vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), P-selectin and L-selectin levels in sera of CRF children and young adults.

Material and methods: The evaluation of adhesion molecule concentrations by ELISA was performed on 15 patients with serum creatinine levels below 265.2 μmol/l (gr. I), 15 patients with serum creatinine levels above 265.2 μmol/l (gr. II) and 15 controls.

Results: sVCAM-1, sICAM-1 and sP-selectin concentrations were elevated in both groups vs controls, whereas sL-selectin levels were decreased in all CRF patients. Mean sVCAM-1 and sICAM-1 values in gr. I and gr. II were comparable. sL-selectin and sP-selectin mean values in gr. II were lower than in gr. I. sICAM-1 correlated with haemoglobin and erythrocyte count in both groups and with haematocrit and serum urea – in gr. I.

Conclusions: Enhanced (sVCAM-1, sICAM-1, sP-selectin) and diminished (sL-selectin) adhesion molecule concentrations in both groups show a state of immunologic imbalance, already present in early stages of CRF. Differences in sL-selectin concentrations between gr. I and II imply a progressive character of CRF-related leukocyte dysfunction. sICAM-1 correlation with anaemia markers may suggest the connection between this molecule and the CRF-related disorders.

Key words: soluble adhesion molecules, immunodeficiency, chronic renal failure, children, young adults.

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Introduction

Chronic renal failure (CRF) and its treatment are associated with complex impairment of the immune system. CRF patients present with acquired immunodeficiency, which is clinically manifested by prolonged skin allograft survival, anergy in cutaneous delayed – type hypersensitivity tests, dysregulated response to vaccinations and increased incidence of infections [1-3]. The latter may result from the insufficient migration of immunocompetent cells, possibly leading to the impaired defence against pathogens.

Integrins, selectins (E-selectin, L-selectin and P-selectin) and cell adhesion molecules from immunoglobulin superfamily, like intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), take part in the subsequent stages of adhesion cascade, determining leukocyte migration to the sites of inflammation or tissue damage [4-7]. Activation of the membrane-bound adhesion molecules, observed during this process, results in their proteolytical shedding from cells into the circulation [8,9]. These soluble forms appear to be biologically active and influence leukocyte attachment to the vascular endothelium, thus playing a crucial role in effective immune response [10,11].

There are studies concerning the immune status of adults with uraemia, but hardly any have dealt with the children population [1-3,12-19]. The aim of the present study was therefore to evaluate serum levels of soluble (s) adhesion molecules: sICAM-1, sVCAM-1, sP-selectin and sL-selectin, in children and young adults with chronic renal failure treated

conservatively. The analysis of selectins, initiating leukocyte rolling, and immunoglobulin superfamily members, responsible for adhesion and diapedesis, was aimed at evaluating the subsequent stages of adhesion cascade. The choice of particular adhesins depended also on the variety of their membrane form location and possible evaluation of endothelial (sICAM-1, sVCAM-1), leukocyte (sL-selectin) and platelet (sP-selectin) activity. We also searched for relationship between circulating adhesin levels and markers of renal function impairment as well as CRF-related complications like anaemia or disturbances in calcium-phosphate metabolism.

Material and methods

The study included 30 children and young adults (15 boys and 15 girls) with CRF, treated conservatively in 2 Departments of Paediatric Nephrology in Wroclaw and Zabrze. Their age ranged from 18 months to 22 years (mean 10.5 years). Mean time of therapy, since the diagnosis of CRF has been established, was 5.5 years (range 3 months – 14 years). Primary diseases causing CRF were: reflux nephropathy (n=11), chronic glomerulonephritis (n=4), urinary tract obstruction (n=3), neurogenic bladder (n=3), amyloidosis (n=2), polycystic kidney disease (n=2), haemolytic uraemic syndrome (n=1), cystynosis (n=1), dysplasia (n=1) and unknown (n=2). CRF patients were divided into two subgroups based on the extent of renal function impairment. Group I included 15 children (9 girls, 6 boys) aged 18 months – 18 years (mean 11.5 years) with mean serum creatinine level 185.6 ± 44.2 μmol/l (range 106.0 μmol/l – 265.2 μmol/l). Group II consisted of 15 children (6 girls, 9 boys) aged 20 months – 22 years (mean 10.1 years) with mean serum creatinine level 512.72 μmol/l ± 256.36 μmol/l (range 265.2 μmol/l – 1281.8 μmol/l). In both groups urinary tract abnormalities were the dominant cause of CRF. The glomerular filtration rate (GFR) was estimated by the Schwartz formula. GFR ranged from 50 to 15 ml/1.73m^2/min in gr. I and was less than 15 ml/1.73m^2/min in gr. II. The control group contained 15 children (10 boys, 5 girls, mean age 11.5 years) with a diagnosis of urinary tract abnormalities or urolithiasis, with normal kidney function. Detailed characteristics of examined children are shown in *Tab. 1*. When necessary, phosphate binders and vitamin D analogue supplementation was applied. None of the patients showed clinical evidence of infection, had malignancies, received recombinant erythropoetin, took antibiotics, or immunosuppressive therapy. Informed consent was obtained from the subjects and their parents.

In each patient, sICAM-1, sVCAM-1, sP-selectin and sL--selectin plasma levels were evaluated. Blood for the examination was drawn from peripheral vein during routine control. Samples were centrifuged at 4°C, 2000 g , for 10 minutes, then serum was stored at -20°C until assay. Serum concentrations of adhesion molecules were determined by commercially available ELISA kits (R&D Systems, Inc., Minneapolis, Minn., USA) on Statfax 2100 (Analco). Each sample was measured in duplicate and the arithmetic mean was considered as a final result. Results were calculated by reference to standard curves. Limits of detection, intra- and interassay variations for adhesion molecules were as follows: sVCAM-1 – 2 ng/ml, 3.5%, 7%; sICAM-1 – 0.35 ng/ml, 3.5%, 7.5%; sP-selectin – 0.5 ng/ml, 4%, 8%; sL-selectin – 0.3 ng/ml, 4%, 8%.

The following investigations were also carried out: renal function evaluation (serum urea and creatinine levels), haematocrit, haemoglobin concentration, peripheral blood erythrocytes, leukocyte, platelet and lymphocyte count, serum total proteins, total cholesterol, triglicerides, calcium, inorganic phosphate.

Results are expressed as mean values ± SD. Differences between all groups were evaluated by using non-parametric tests (Kruskall-Wallis, Mann-Whitney U). Correlations between variables were evaluated by Spearman's correlation coefficient. Statistical analysis was performed using the package Statistica 5.0 (StatSoft). A p value < 0.05 was considered significant.

Results

Baseline laboratory test results are shown in *Tab. 1*.

Serum sVCAM-1 levels

The concentrations of sVCAM-1 were increased in all CRF patients vs controls, irrespective of their serum creatinine levels (*Tab. 2*). No significant difference between sVCAM-1 concentrations in group I and group II was observed. The levels of sVCAM-1 correlated with granulocyte count ($r = 0.58$; $p < 0.05$) in group I, with lymphocyte count ($r = -0.57$; $p<0.05$) and platelet count ($r = -0.53$; $p<0.05$) – in group II.

Serum sICAM-1 levels

The concentrations of sICAM-1 were elevated in CRF patients from both groups, when compared with controls (*Tab. 2*). There were no significant differences between sICAM-1 concentrations in group I and group II. Strong linear correlations were found between sICAM-1 levels and serum concentrations of: haemoglobin ($r = -0.83$; $p < 0.001$), haematocrit ($r = -0.86$; $p < 0.001$), erythrocyte count ($r = -0.82$; $p < 0.001$), urea ($r = 0.70$; $p < 0.01$) and granulocyte count ($r = -0.71$; $p < 0.01$) – in group I; haemoglobin ($r = -0.66$; $p < 0.01$) and erythrocyte count ($r = -0.53$; $p < 0.05$) – in group II.

Serum sP-selectin levels

The levels of sP-selectin were elevated in all CRF patients, when compared with controls (*Tab. 2*). sP-selectin concentrations in group I were significantly higher than in group II. The levels of sP-selectin correlated with alkaline phosphatase activity ($r = 0.74$; $p < 0.001$) in group I and with lymphocyte count ($r = 0.59$; $p < 0.05$) in group II.

Serum sL-selectin levels

The levels of sL-selectin were decreased in all CRF patients vs controls, irrespective of their serum creatinine levels (*Tab. 2*). Additionally, sL-selectin concentrations in group II were significantly lower than in group I. No correlation was found between sL-selectin and biochemical markers in any of the analyzed groups.

Table 1. **CRF patients characteristics and baseline laboratory test results presented as mean ± SD**

Results	I GROUP n = 15	II GROUP n = 15
Age (years)	11.5 ± 4.4	10.1 ± 5.7
CRF duration (months)	72 ± 53.8	63.8 ± 53.8
Haematocrit (%)	36.3 ± 6.0	29.8 ± 3.7
Haemoglobin (g/dl)	12.4 ± 2.1	10.0 ± 1.3
Erythrocytes (T/l)	4.3 ± 0.6	3.4 ± 0.5
Leukocytes (G/l)	8.1 ± 2.5	6.7 ± 2.0
Neutrophils (%)	57.2 ± 9.0	55.5 ± 11.6
Lymphocytes (%)	34.1 ± 7.7	37.9 ± 12.7
Trombocythes (G/l)	309.1 ± 94.9	297.1 ± 107.6
Sodium (mmol/l)	142.1 ± 4.7	140.9 ± 3.8
Potassium (mmol/l)	4.4 ± 0.8	4.5 ± 0.9
Calcium (mmol/l)	2.25 ± 0.5	2.38 ± 0.4
Phosphate (mmol/l)	1.49 ± 0.52	1.71 ± 0.46
Urea (mmol/l)	85.8 ± 43.5	132.0 ± 47.0
Creatinine (μmol/l)	186.3 ± 40.7	511.0 ± 256.4
Total proteins (g/l)	73.2 ± 12.7	70.7 ± 0.9
Total cholesterole (mmol/l)	5.59 ± 1.7	5.51 ± 1.3
Triglicerydes (mmol/l)	21.2 ± 13.7	16.2 ± 8.9

Table 2. **Mean serum sVCAM-1, sICAM-1, sP-selectin and sL-selectin concentrations in examined groups**

Parameter [ng/ml] Mean ± SD	Control group n = 15	CRF – group I n = 15 serum creatinine <265.2 μ mol/l	CRF – group II n = 15 serum creatinine >265.2 μ mol/l	Differences between controls, gr. I and gr. II (Kruskall-Wallis test)	Differences between controls and gr. I (Mann-Whitney U test)	Differences between controls and gr. II (Mann-Whitney U test)	Differences between gr. I and gr. II (Mann-Whitney U test)
sVCAM-1	625.00 ± 80.00	1442.00 ± 711.03	1478.67 ± 559.76	p = 0.0000	p = 0.0000	p = 0.0000	p = 0.6600
sICAM-1	231.60 ± 60.87	334.93 ± 109.56	348.67 ± 82.27	p = 0.0006	p = 0.0042	p = 0.0002	p = 0.7400
sP-selectin	93.13 ± 14.59	189.33 ± 61.13	140.13 ± 55.28	p = 0.0002	p = 0.0000	p = 0.0238	p = 0.0279
sL-selectin	6492.66 ± 1914.45	1982.67 ± 391.51	1618.00 ± 484.93	p = 0.0000	p = 0.0000	p = 0.0000	p = 0.0327

Discussion

Deleterious effects of uraemia may change various components of immune system. In our study, the analysis of soluble cell adhesion molecules in children with chronic renal failure treated conservatively revealed the elevation of sVCAM-1, sICAM-1 and sP-selectin serum concentrations. Bonomini et al. [20] also found increased serum levels of sICAM-1, sVCAM-1, sE-selectin and sP-selectin in adult patients with CRF on conservative treatment and on maintenance dialysis. In the predialysis group circulating adhesion molecule levels correlated positively with serum creatinine levels. Likewise, Mrowka et al. [21] described the increased concentrations of sICAM-1 and sVCAM-1, strongly correlating with serum creatinine levels, in patients with CRF treated conservatively, as well as in the dialyzed ones. In both studies, the authors pointed out impaired excretory kidney function and inefficient elimination of molecules as the main causes of their accumulation. In our patients, we only noticed the relationship between sICAM-1 and serum urea levels in the group of patients with serum creatinine concentration below 265,2 μmol/l. Ara et al. [22] did not confirm the association between sICAM-1, sVCAM-1, sE-selectin, or sP-selectin and serum creatinine levels in CRF patients either. Taking into account the fact that sICAM-1 and sVCAM-1 concentrations in our study did not increase as renal insufficiency progressed, we may speculate that various overlapping mechanisms may be responsible for elevated concentrations of those molecules. It was documented that the activity of pro-inflammatory cytokines (e.g. TNF-α) is enhanced in uraemia [23,24]. Pigott et al. [9] revealed the elevation of sICAM-1 and sVCAM-1 concentrations due to stimulation by TNF-α. Thus, the increased levels of sVCAM-1 and sICAM-1 in our patients may result from TNF-α stimulation. Correlations observed between sICAM-1 and markers of anaemia need to be further investigated.

sP-selectin, elevated in all our patients, can be derived from both platelets and endothelium. The study by Gamble et al. [25] revealed that sP-selectin inhibits the adhesion of neutrophils, previously activated with TNF-α, to the endothelium. That reaction weakens the effectiveness of further leukocyte migration. The latter was also confirmed by Nagata et al. [26], who observed the inhibition of leukocyte superoxide anion production in the presence of recombinant, circulating P-selectin. Our study [27] showed that the higher serum creatinine concentration, the more severe peroxidative damage in CRF children. Taking into account these results and our

observations, we hypothesized that enhanced concentrations of sP-selectin may be regarded as an indicator, reflecting disordered leukocyte-endothelial adhesion and impaired production of active oxygen compounds in uraemia.

Among soluble adhesion molecules, sL-selectin was the only one to show decreased serum concentrations in adults with CRF [22,28]. Our results confirmed diminished levels of sL-selectin also in children. Dou et al. [28] explained this phenomenon by impaired granulocyte function in uraemia, which resulted in reduced adhesion molecule synthesis. Schleiffenbaum et al. [11] and Kawabata et al. [29] compared the expression of membrane receptors for L-selectin with the levels of its soluble form. They emphasized that the presence of sL-selectin in serum is a consequence of its release from the surface of activated leukocytes. Schleiffenbaum et al. [11] also showed that sL-selectin binds to L-selectin counterreceptors on endothelium, thus inhibiting the adhesion of leukocytes to the endothelium. Therefore, lowered sL-selectin concentrations result from either leukocyte dysfunction and failure to shed sL--selectin, or from sL-selectin binding to endothelial receptors. Consequently, decreased sL-selectin concentrations reflect the impairment of leukocyte function and migration, that may be, at least in part, responsible for increased incidence of infections in CRF children. Moreover, our examination revealed that sL-selectin levels in patients with serum creatinine levels above 265.2 μmol/l were lower than those in patients with the values below 265,2 μmol/l. Nonetheless, sL-selectin concentrations decrease as renal function deteriorates, thus showing progressive granulocyte impairment. In contrast, Dou et al. [28] did not observe correlation between sL-selectin concentration and expression of L-selectin on granulocytes. They suspected that the altered balance between sL-selectin synthesis and elimination may be responsible for its reduced activity in patients with CRF.

In conclusion, enhanced (sICAM-1, sVCAM-1, sP--selectin) and simultaneously diminished (sL-selectin) adhesion molecule concentrations document a state of imbalance, already appearing in children and young adults with mild CRF. Differences in sL-selectin levels between examined groups show the progressive character of leukocyte dysfunction in CRF patients. Relationships between sICAM-1 and markers of anaemia suggest the role of this molecule in disturbances observed in CRF patients. However, the latter needs further detailed investigation.

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Can von Willebrand factor, platelet-endothelial cell adhesion molecule-1 and thrombomodulin be used as alternative markers of endothelial cell injury in human glomerulonephritis?

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Abstract

Purpose: **There is growing evidence that endothelial cells (EC) are active participants of an inflammatory process in glomeruli.**

Material and methods: **We compared the glomerular expression of three EC-coupled molecules, i.e. platelet-endothelial cell adhesion molecule-1 (PECAM-1 or CD31), von Willebrand factor (vWF) and thrombomodulin (TM) in 60 patients with glomerulonephritis (GN) and five normal kidneys (NK). The alkaline phosphatase anti-alkaline phosphatase method was used to examine the expression of these proteins in the biopsy specimens.**

Results: **In NK, the expression of CD31 and vWF comprised the whole glomerular network. In contrast, the expression of TM was much lower and localized mainly to EC at the vascular pole and adjacent areas. In GN, the glomerular staining for CD31 and vWF was significantly reduced. A fall in the expression of both these EC antigens was more pronounced in proliferative forms of GN (PGN) than in non-proliferative GN (NPGN) (CD31: NPGN vs. PGN, $p < 0.02$; vWF: NPGN vs. PGN, $p < 0.05$). In addition, a linear relationship between the expression of CD31 and vWF was found in GN ($r = 0.8$, $p < 0.001$). Conversely to CD31 and vWF, a marked increase in glomerular reactivity for TM was observed in all the patients with GN (GN: 2.12 ± 0.32, NK: 0.95 ± 0.05, $p < 0.02$). However, the highest expression of TM was found in membranoproliferative GN and lupus GN.**

Conclusions: **Our results suggest that CD31 and vWF may be used as markers of glomerular EC loss during GN, whereas TM staining seems to reflect EC activation in response to circulating and/or released in situ procoagulant factors.**

Key words: von Willebrand factor, platelet-endothelial cell adhesion molecule-1, thrombomodulin, glomerulonephritis.

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Introduction

Injury of the glomerular capillary network with endothelial cells' (EC) damage has been shown to play an important role in the progression of glomerulonephritis (GN) [1]. Shimizu et al. have demonstrated a marked loss of EC in glomeruli in experimental rapid progressive GN, using an antibody to thrombomodulin (TM) as the marker of EC [2]. The same findings were observed in rats with anti-Thy 1.1 GN when an antibody to platelet-endothelial cell adhesion molecule-1 (PECAM-1 or CD31) was applied to stain EC [3,4]. The authors of the above studies suggest that damage to the glomerular EC with the following incomplete repair is an important factor in the appearance and progression of the glomerular sclerosis [2-4].

Others and we have previously demonstrated the loss of glomerular EC also in various morphological forms of human GN with CD31 used as the marker of EC [5-7]. Results concerning the expression of TM in human GN are, however, inconsistent. Increased expression of TM has predominantly been shown in glomeruli of patients with lupus GN (LGN) [8,9]. In this context, Frijns et al. have observed significantly higher plasma levels of soluble TM and von Willebrand factor (vWF) in patients with a history of lupus glomerulonephritis LGN than in systemic lupus erythematosus (SLE) patients without GN [10]. The authors have suggested that the increase in these EC markers reflects a state of persistent EC activation specifically localized in the kidneys. Woywodt et al. [11] used antibodies against vWF

and CD31 in order to isolate EC from peripheral blood of patients with ANCA-associated vasculitis and came to the same conclusion. In primary GN, the highest expression of TM, compared to other forms of glomerular diseases, has been observed in membranoproliferative GN (MPGN) [8]. On the other hand, higher intraglomerular staining for TM has been reported in the remission phase of focal-segmental glomerulosclerosis (FSGS) than in the nephrotic stage of this disease [12].

Although PECAM-1, vWF and TM can serve as EC markers, their localization in the cell [13,14] and function [13, 15-17] differ from each other. PECAM-1 is one of the adhesion molecules of 130 kDa and belongs to the immunoglobulin (Ig) superfamily. It is present in thrombocytes, lymphocytes, neutrophils, and EC, in which it concentrates at the intercellular borders of adjacent cells. Its redistribution depends on cytokines, such as INF-γ and TNF-α [14]. Its ligands include homophilic interactions with itself, $\alpha v\beta 3$ integrins and CD38 [18]. It has been implicated in various biological functions such as modulation of integrin-mediated cell adhesion, angiogenesis, apoptosis, cell migration, negative regulation of immune signaling, autoimmunity, macrophage phagocytosis, IgE-mediated anaphylaxis, and thrombosis [18]. CD31 plays an important role in transendothelial migration of leukocytes without influence on adhesion of these cells to endothelium [15].

TM is a thrombin receptor present on the surface of the EC in arteries, veins, capillaries, lymphatic vessels, platelets, and placental syncytiotrophoblast cells. It is considered to be the main factor taking part in the control of thrombogenesis [16]. TM acts as a cofactor for the activation of plasma protein C. EC membrane-bound TM forms a high-affinity complex with thrombin, and inhibits thrombin interaction with fibrinogen and protease-activated receptor. TM-thrombin complex is also a potent activator of protein C that enhances thrombin-dependent protein C activation [17]. TM-thrombin complex activates also latent plasma carboxypeptidase B, which removes carboxy-terminal arginine and lysine residues from fibrin. These residues are important for the sequestration of fibrynolytic enzymes on the fibrin matrix, and their removal renders the clot more resistant to lysis [19]. In vitro, exposure of EC to neutrophils or hydrogen peroxide promotes the release of TM. Soluble TM consists of fragments of various molecular weights, which are most likely either proteolytically degraded from cellular TM or derived from the injured EC [20,21].

Another marker of EC is vWF [22]. It is a multimeric plasma glycoprotein with the molecular weight of 270 kDa. This glycoprotein mediates platelet adhesion to injured vessel walls and serves as a carrier and stabilizer for coagulation factor VIII, as well as it has functional binding domains to platelet glycoprotein Ib, glycoprotein IIb/IIIa, collagen and heparin. It is present in EC, platelets, and megakaryocytes, as well as in numbers of tumors including hemangiomas, hemangiosarcomas and Kaposi's sarcomas [22].

In the light of the above data, it seemed reasonable to address the question whether all these three EC markers can alternatively be used for EC visualization in an inflamed glomerulus. Our results show a variable loss of the glomerular staining for CD31 and vWF in renal specimens with GN. On the other hand, an increased immunoreactivity for TM is observed in GN.

Material and methods

Sixty patients with biopsy-proven GN were enrolled into the study. The morphological diagnosis was based on conventional light microscope and immunopathological examinations. Biopsy material remaining after the latter investigation was used in this study. Fourty-five patients with primary GN had proliferative forms of GN (PGN). Among them, 41 had mesangial proliferative GN (MesPGN) while membranoproliferative GN (MPGN) was diagnosed in four. In the group with non-proliferative glomerulopathies (NPGN), seven patients had idiopathic membranous GN (IMGN) and four had focal-segmental glomerulosclerosis (FSGS). Out of four patients with lupus nephritis (LGN), three were categorized as PGN (1 patient, class III; 2 patients, class IV) and one patient having class V of glomerular lesions was classified to the NPGN group. Clinical and morphological data of patients are presented in *Tab 1*.

The expression of TM, CD31 and vWF was examined by immunohistochemistry on acetone-fixed sections using the three-step alkaline phosphatase – anti-alkaline phosphatase (APAAP) method as previously described [7]. Monoclonal antibodies against TM (Dako Corporation, Glostrup, Denmark), and CD31 (Novocastra, Newcastle, UK) were used, whereas a plyclonal antibody to vWF (Novocastra) was applied. The expression of CD31 was examined in 46 cases, that of vWF in 37 subjects and TM in 38 patients. The expression of all these molecules was examined in 19 patients (MesPGN, 14; MPGN, 2; IMGN, 2; LGN, 1), that of CD31 and vWF in 25 subjects (MesPGN, 17; MPGN, 2; IMGN, 4; FSGS, 1; LGN, 1), TM and CD31 in 29 patients (MesPGN, 20; MPGN, 4; IMGN, 2; FSGS, 1; LGN, 2), and TM together with vWF in 26 individuals (MesPGN, 19; MPGN, 2; IMGN, 2; FSGS, 1; LGN, 2). Normal-appearing kidney tissue (n = 5) surrounding the removed tumor served as controls.

Regarding CD31 and vWF, evaluation of the glomerular expression of these EC markers was performed using a following grading scale: grade 3, reflecting the staining intensity (I) in the normal kidney; grade 2, defined as a moderate decrease in the staining; and grade 1, defined as a severe decrease in the staining intensity. With respect to TM, a scale in an opposite direction was used, beginning with the estimation of the staining intensity in normal glomeruli at 1 point and increasing upward to 3 points in specimens with GN. Based on the above grading scales, the mean glomerular expression scores (GES) were calculated for each molecule, according to the formula: $GES = I_{(1)}/100\% + ... I_{(N)}/100\%/N$, where N was the number of glomeruli in the renal specimen. The non-paired Mann-Whitney U test was used to test differences between patients with PGN and NPGN. Finally, a linear regression analysis between the expression CD31, vWF and TM was performed, respectively. The P values less than 0.05 were considered significant.

Results

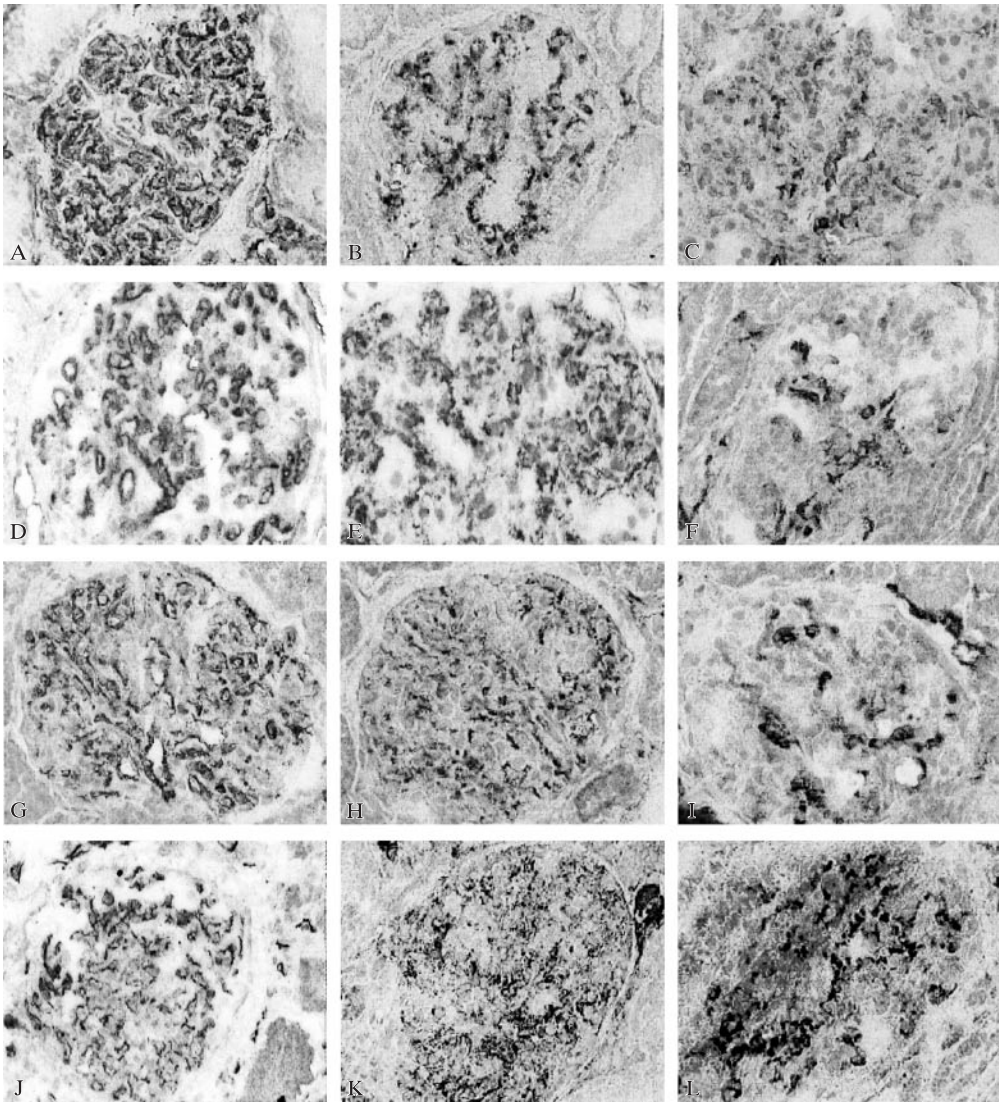
In the normal kidney, the expression of CD31 and vWF comprised the whole glomerular network, although some differ-

Table 1. **Clinical and morphological data of patients at the time of renal biopsy**

Morphology	No of Patients	Age (yr) (Mean ± SD)	Serum Creatinine (μmol/l) (Mean ± SD)	Urinary Protein (g/24 h) (Mean ± SD)
		Primary GN		
Proliferative GN				
MesPGN	41	31.4 ± 15.9	81.4 ± 19.2	2.2 ± 1.3
MPGN	4	52.0 ± 1.0	99.2 ± 18.1	3.7 ± 0.3
Non-proliferative GN				
FSGS	4	24.1 ± 14.1	97.4 ± 20.3	3.4 ± 0.8
IMGN	7	37.3 ± 12.7	82.6 ± 18.4	3.2 ± 1.4
		Lupus nephritis		
Class III	1	29	97.24	3.4
Class IV	2	37.5 ± 17.7	165.7 ± 102.4	3.2 ± 0.7
Class V	1	31	79.6	2.5

Abbreviations are: PGN, proliferative glomerulonephritis; MesPGN, mesangial proliferative GN; MPGN, membranoproliferative GN; NPGN, non-proliferative GN; IMGN, idiopathic membranous GN; FSGS, focal-segmental glomerulosclerosis

Figure 1. **A comparison between glomerular expression of CD31 (A, D, G, J), von Willebrand factor (B, E, H, K), and thrombomodulin (C, F, I, L), respectively, in the normal kidney (A, B, C), idiopathic membranous nephropathy (D, E, F), mesangial proliferative GN in the course of IgA GN (G, H, I), and class IV lupus nephritis (J, K, L)**



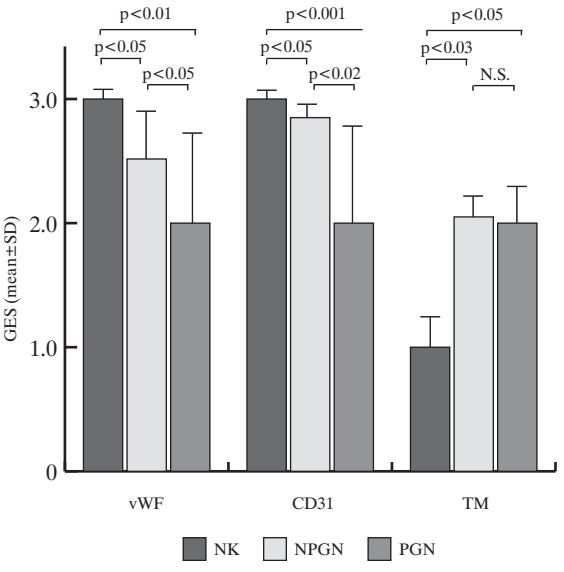
ences in the staining patterns could be noticed (*Fig. 1A* and *1B*). In contrast, small amounts of TM localized to the vascular poles of normal glomeruli (*Fig. 1C*).

In GN, some reduction in the expression of CD31 and vWF was observed in biopsies with NPGN (*Fig. 1D* and *1E*). It was related with the appearance of sclerotic alterations in glomeruli. On the other hand, a variable decrease in the expression of both these EC markers, dependent on the degree of the proliferative response of glomerular cells, was noted in PGN. Thus, the immunoreactivity for CD31 and vWF was mildly reduced in IgA-GN with moderate mesangial cell proliferation (*Fig. 1G* and *1H*), but markedly diminished in biopsies with severe proliferative lesions in MPGN and class IV lupus nephritis (*Fig. 1J* and *Fig. 1K*). In particular, the lowest values of GES for CD31 and vWF were observed in MPGN (CD31: 1.43 ± 0.64, $p < 0.02$ vs. IMGN and $p < 0.01$ vs. the whole NPGN group; vWF: 1.2 ± 0.0).

In contrast to the decreasing trend in the expression of CD31 and vWF, an increased glomerular reactivity for TM was a general feature of GN (*Fig. 1F*, *1I*, *1L*). However, the highest values of GES for TM were obtained in MPGN and LGN (2.36 ± 0.46 for both groups of patients).

Results of a semiquantitative evaluation of the glomerular expression of CD31, vWF and TM in NPGN and PGN, in comparison to the normal kidney, are shown in *Fig. 2*. As demonstrated in *Fig. 3*, a linear correlation between the glomerular expression of CD31 and vWF could be stated in the whole population of patients examined. No relationship between the glomerular expression of CD31 and TM, as well as vWF and TM could be observed in GN.

Figure 2. **Evaluation of glomerular expression of von Willebrand factor (vWF), CD31, and thrombomodulin (TM) in normal kidneys (NK), non-proliferative GN (NPGN), and proliferative GN (PGN). GES: mean glomerular expression score**



Discussion

Our study showed marked differences between the glomerular expression of TM, CD31 and vWF in the normal kidney. The expression of CD31 and vWF comprised the whole glomerular network, whereas the immunoreactivity for TM localized only to the vascular poles of the glomeruli. With respect to the distribution of CD31 in the normal glomeruli, Wada et al. have reported similar results in the rat kidney [3] and Sivridis et al. in the human kidney [6]. Also regarding the pattern of reactivity for TM in the normal kidney, previous results of Mizutani et al. [8] and Tomura et al. [9], are in line with these presented in this study.

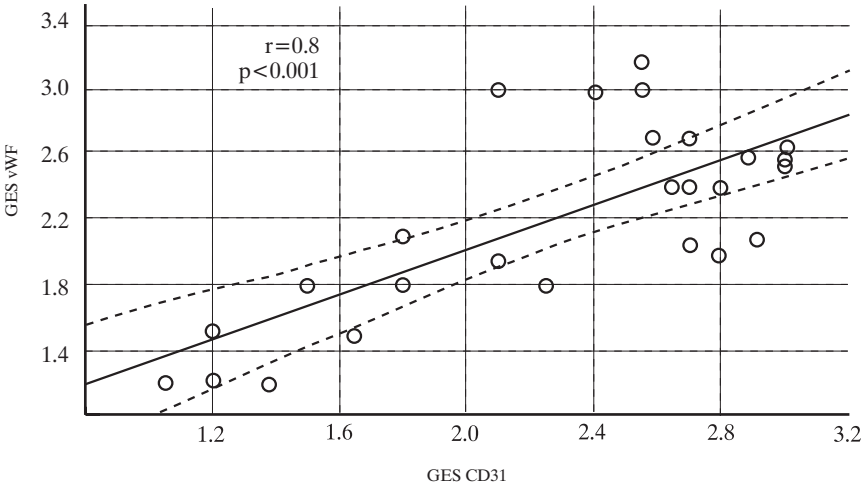
Not only did the expression of the examined proteins differ with respect of their pattern of reactivity in the normal kidney, but also in terms of their expression in different types of GN. Most of our patients presented features of MesPGN. Previously, we have observed a variable loss of CD31 staining in the course of IgA nephropathy [7]. This study demonstrates that the deepest fall in the expression of CD31 is found in MPGN, where mesangial cell proliferation largely exceeds that observed in MesPGN. Thus, in PGN, the reduction in the expression of CD31 seemed to be dependent on the extent of proliferative response of mesangial cells and subsequent destruction of the glomerular architecture.

Experimental studies on acute models of GN in which PECAM-1 has been used as the marker of EC show that these cells have a great regenerative capacity [3,4]. However, in models of progressive glomerular injury, loss of PECAM-1 staining signified impairment of capillary regeneration and preceded the development of glomerulosclerosis [3,4]. The expression of CD31 could particularly not be found in areas occupied by macrophages [4]. Since PECAM-1 has been shown to regulate the transmigration of neutrophils and monocytes across EC monolayer [15,23], loss of its expression may favor the leukocyte accumulation inside the glomerulus, which is the classical feature of the proliferative forms of GN [24,25].

Compared to MesPGN and MPGN, reduction in the glomerular expression of CD31 in our patient population with NPGN, i.e. in IMGN and FSGS, was relatively low. These results are in disagreement with that presented recently by Sivridis et al. [6]. In the above study, complete loss of the glomerular expression of PECAM-1 was observed in 9 out of 15 cases with IMGN. It may be supposed that more advanced sclerotic lesions in glomeruli were responsible for the discrepancy between the results of both studies. Since ten of these patients had higher values of serum creatinine concentration than our subjects with IMGN, such an explanation seems to be reasonable [6].

In our study, the glomerular expression of CD31 was related with that of vWF. Although vWF has bee used as a marker of EC in chronic allograft nephropathy [26], its glomerular expression in GN has not been demonstrated until now. As in the case of CD31, the more pronounced the distortion of glomerular architecture, the lower expression of vWF was observed. Recently, increased urinary excretion of vWF, including its functionally active form, has been reported in patients with active lupus nephritis [27]. Intriguingly, the highest levels of urinary vWF were found in patients with rapid progressive forms of LGN, who presented with the same range of proteinuria as patients with nephrotic syndrome without rapid progressive renal fail-

Figure 3. **Results of the linear regression analysis between the glomerular expression of CD31 and von Willebrand factor (vWF) in the whole population of patients examined. GES: mean glomerular expression score**



ure. In this context, increased levels of vWF have also been found in sera of patients with history of LGN [10]. The authors suggested that both increased serum concentration and urinary excretion of vWF in LGN might be a marker of local renal EC damage, which reflected the severity of immune inflammation and intravascular coagulation [10,27]. Taking into account our results and the fact that vWF plays a pivotal role in platelet adhesion and aggregation and acts as a ligand protein to support endothelial cell adhesion [22], this supposition sounds as a plausible hypothesis. However, larger population of patients with MPGN and, particularly, with LGN should be examined to draw final conclusions.

In contrast to CD31 and vWF, our study showed an increased glomerular reactivity for TM in patients with GN. Intriguingly, the glomerular expression of TM did not statistically differ between patients with NPGN and PGN. These results do not correspond with previously reported studies on the expression of TM in GN [8,9]. Although a moderate staining for TM has been observed in IMGN and minimal change disease, a significant increase in its expression has primarily been observed in idiopathic MPGN and LGN [8,9]. In the study of Mizutani et al., a direct correlation between the degree of TM expression and amounts of subendothelial immune deposits in LGN and MPGN has been found. In contrast, the expression of TM was not related with the presence of subepithelial or mesangial immune deposits in IMGN and IgA nephropathy, respectively [8]. Though, Tomura et al. have noticed no apparent differences in the intensity and distribution of TM staining among the different morphological forms of LGN [9]. Furthermore, the appearance of TM staining in the remission phase of FSGS has been found in another study. The authors of this study has linked the emergence of TM staining with the recovery from EC damage and suggested TM involvement in the repair process in GN [12]. The latter assumption might be strengthen by the fact that intravenous administration of recombinant human soluble TM to the rats with thrombotic rapid progressive GN had not only anti-thrombotic effects, but also attenuated leukocyte infiltration into the glomerulus [28].

In the light of these data, TM appears to be not only an anti-thrombotic but also anti-inflammatory molecule. The emergence of its staining in GN could be interpreted as an EC trial to maintain the local homeostasis and prevent the progression of glomerular lesions. In the latter context, the association of the highest expression of TM and the lowest one of CD31, observed in our patients with MPGN and LGN, might reflect maximal EC activation, which is, however, insufficient under condition of continuing EC injury.

Conclusions

Our results suggest that the reduction in the glomerular expression of CD31 and vWF in GN seems to reflect EC loss as a result of the inflammatory process inside the glomerulus. On the other hand, increased expression of TM points out to the active participation of glomerular EC in mechanisms aimed at the preservation of the glomerular architecture.

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Kidney function estimated with the different formulas in centenarians

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Abstract

Purpose: **There are growing doubts about the accuracy of Cockcroft-Gault formula (CG) used for the estimation of creatinine clearance, especially in elderly. Recently, the authors of the multicenter trial of the Modification of Diet in Renal Diseases (MDRD) have proposed a new equation. Moreover, Baracskay et al. (B), proposed the special formula for the estimation of kidney function (KF) in elderly. The aim of our study was to compare the results of KF calculated with these three formulas in centenarians.**

Material and methods: **The study involved 50 centenarian subjects aged 100-111 years (41 females and 9 males) who participated in Polish Centenarians Program. In all of them KF was estimated with the CG, B and MDRD formulas.**

Results: **In the whole population examined, the mean KF according to CG was lower in comparison to both others ($p < 0.001$ vs both B and MDRD). Also, in females CG results were the lowest ($p < 0.001$ vs both B and MDRD). In contrast, KF calculated according to CG and B did not differ in males. The results of the MDRD formula significantly exceeded the two others also in males ($p < 0.001$ vs CG and B). No impact of gender on the obtained results could be found when CG and MDRD were used. However, according to B, the mean values for females were higher ($p < 0.01$).**

Conclusions: **KF calculated with the CG, B and MDRD formulas significantly differed in the centenarians examined. Thus, further studies, which include a reference standard, are necessary to answer the question which of these mathematical formulas is the most reliable for the calculation of KF in the elderly.**

Key words: centenarians, kidney function, mathematical formulas.

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Introduction

In elderly, all accepted methods for the estimation of kidney function (KF) are neither precise nor accurate. Since the doses of many drugs should be adjusted for KF, its accurate assessment is crucial in this group of patients [1]. The measurement of creatinine clearance is difficult to carry out due to the increasing with age problem with proper 24 h urine collection [2]. In addition, decline in the muscle mass during the aging may keep creatinine in the normal range even after significant fall in the glomerular filtration rate (GFR) [3].

Thus, mathematical formulas are often used to assess KF in elderly individuals. The most popular one is the Cockcroft--Gault formula [4]. However, due to large body of evidence about its inaccuracy [2,5-7] new equations have been proposed [8,9]. Baracskay et al. [8] proposed the special formula for the estimation of KF in elderly. Recently, the authors of the multicenter trial of the Modification of Diet in Renal Diseases (MDRD) created a new one that is recommended as the most accurate estimation of GFR [9].

The aim of our study was to compare the results of KF calculated using these three formulas in centenarians.

Material and methods

The study is part of the Polish Centenarians Program (coordinated by the International Institute of Molecular and

Figure 1. **Kidney function estimated with three different formulas (CG – Cockcroft-Gault formula, B – Baracskay et al. formula, MDRD – The Modified Diet of Renal Diseases formula)**

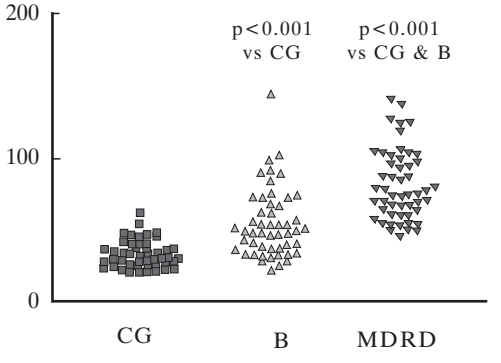
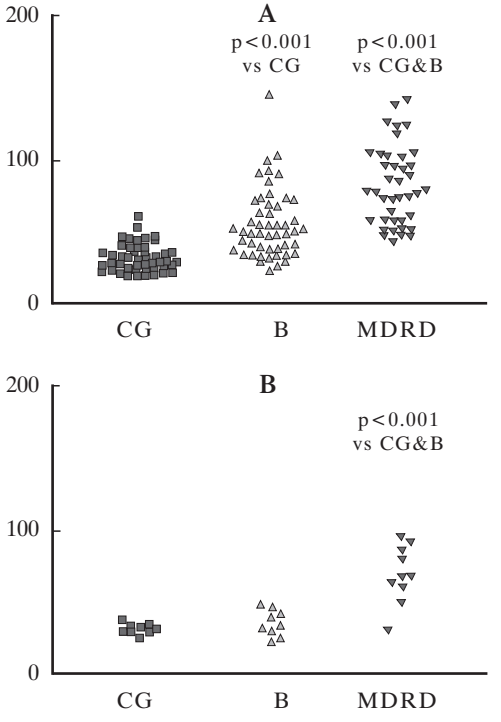


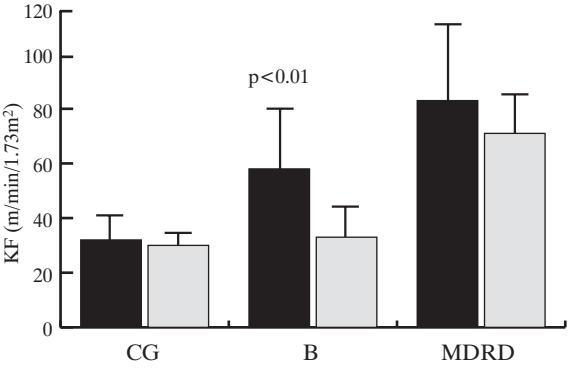
Figure 2. **Kidney function estimated with three different formulas in females (A) and males (B) (CG – Cockcroft-Gault formula, B – Baracskay et al. formula, MDRD – The Modified Diet of Renal Diseases formula)**



Cell Biology in Warsaw) that was developed to assess the environmental and genetic factors associated with aging in Poland. The details of the study were presented elsewhere [10]. The study was based on a questionnaire and on the analysis of the standard morphological and biochemical parameters, which were performed at the Central Laboratory of the Warsaw Medical Academy (Warsaw, Poland).

The study involved 50 centenarian subjects (41 females and 9 males) aged 100-111 years. The mean age of studied subjects was 101.7 ± 2.0 years. All these individuals had serum creatinine within the normal range.

Figure 3. **The mean kidney function estimated with three different formulas in females (white bars) and males (gray bars) (CG – Cockcroft-Gault formula, B – Baracskay et al. formula, MDRD – The Modified Diet of Renal Diseases formula)**



In all of them, KF was estimated with three different formulas. The formulas are presented below:

Cockcroft and Gault formula (CG) [4]:

CG = (140 – age)/(72 x PCr(mg/ml), for females multiplied by 0.85

Baracskay et al. formula (B) [8]:

B= ½[100/PCr(mg/ml)] + 88 – age)

MDRD formula [9]:

MDRD=186 x $PCr^{-1.154}$ x $age^{-0.203}$, for females multiplied by 0.742.

The obtained results were adjusted for body surface area (BSA), which was calculated in each subject based on the Dubois and Dubois method [11].

Statistical analysis

The results are presented as mean ± SD. Statistical analysis was performed with t-Student's test or Kruskal-Wallis one-way ANOVA, as appropriate. A p values less than 0.05 were considered to be significant.

Results

The mean KF according to CG was lower than calculated with the two other methods (CG, 33.4 ± 9.0 ml/min/1.73m^2; B, 55.5 ± 23.5 ml/min/1.73m^2; B vs CG $p < 0.001$; MDRD, 83.9 ± 30,0 ml/min/1.73m^2; $p < 0.001$ vs both CG and B) (*Fig. 1*). In females, values calculated using the CG formula were lower than obtained with both B and MDRD equations (CG, 33.6 ± 9.8 ml/min/1.73m^2; B, 59.5 ± 23.7 ml/min/1.73m^2; B vs CG, $p < 0.001$; MDRD, 85.9 ± 31.9 ml/min/1.73m^2; $p < 0.001$ vs CG and B) (*Fig. 2A*). In males, KF assessed with CG and B was comparable (CG, 32.0 ± 3.3 ml/min/1.73m^2; B, 36.3 ± 8.8 ml/min/1.73m^2. However, values obtained with the MDRD formula were higher (73.9 ± 15.2 ml/min/1.73m^2; $p < 0.001$ vs CG and B) (*Fig. 2B*).

No impact of gender on the obtained results could be found when CG and MDRD were used for the assessment of KF (CG: females, 33.6 ± 9.8 ml/min/1.73m^2; males, 32.0 ± 3.3 ml/min/1.73m^2; MDRD: females, 85.9 ± 31.9 ml/min/1.73m^2;

males, 73.9 ± 15.2 ml/min/1.73m^2). Though, KF calculated with the B formula was significantly higher in females than in males (59.5 ± 23.7 ml/min/1.73m^2 and 36.3 ± 8.8 ml/min/1.73m^2, $p < 0.01$) (*Fig. 3*).

Discussion

In the absence of a simple and accurate method for the assessment of KF, to estimate it mathematical formulas are used. With the aim to find the best one, the results obtained with different formulas are often compared. However, as far as we know there is no data about the usage of different formulas in centenarians. We decided to compare the results of KF calculated with the three mostly used formulas in subjects aged 100 years and more.

One has to realize that the CG formula is used for the assessment of creatinine clearance (C_{cr}), whereas both B and MDRD equations estimate the GFR [4,8,9]. Due to these differences we decided to define calculated values as KF. According to Fliser et al. [5], significant differences between the values of C_{cr} and GFR could particularly be observed in the older population. In our study, the results obtained with CG were the lowest. This could suggest that the CG formula underestimates KF in centenarians.

The results obtained using the equation of Baracskay et al. [8] showed the difference between females and males. The difference was probably noticed due to the fact that this equation, as the only one known, has no multiplier for women. The Baracskay et al. formula was created based on the measurement of KF in elderly subjects with iothalamate clearance (^{125}I iothalamate) [8]. The study involved only 41 subjects and these were predominantly females (32 females and only 9 males). Since the number of males was very small, it seems obvious that the sex impact on the GFR was not noticed. We have already reported on the difference between the results calculated using CG and B in centenarians [12]. As far as we know, the formula of Baracskay et al. [8] has never been validated on a bigger group of elderly subjects.

In the present study, KF calculated according to the MDRD formula was the highest one (*Fig. 1 & 2*). The MDRD formula was created based on the results of ^{125}I-iothalamate clearance in 1070 patients aged 18-70 years [9]. Because there were no very old subjects in the examined group, the accuracy and precision of this formula for such a population was not validated at all. Recently, Lamb et al. [13] have demonstrated that in subjects aged 65-92 years the MDRD equation may slightly overestimate the GRF.

In summary, our results showed that KF calculated with the CG, B and MDRD formulas significantly differed in the centenarians examined. Thus, further studies, which include a reference standard, are necessary to answer the question which of these mathematical formulas is the most reliable for the calculation of KF in the elderly.

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Prooxidant-antioxidant balance in blood during the surgical treatment of obliterating arterial atherosclerosis in the lower extremities

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Abstract

The chronic disorder of arterial circulation in the lower extremities due to the atherosclerotic injuries of the femoral-popliteal-tibial arteries was accompanied by the shift of the blood prooxidant-antioxidant balance towards more active free radical oxidation and by a depletion of antioxidant system. After restoring arterial blood flow in the ischemic lower extremities this balance was shifted towards more prominent lipid peroxidation processes. Such pattern of prooxidant-antioxidant balance changes implicates the development of strategies for its correction targeted on the reduction of lipid peroxidation activity and therefore on a tissue defense against the reperfusion injury.

Key words: obliterating atherosclerosis, blood prooxidant-antioxidant balance, reperfusion-reoxygenation injury.

Introduction

The recovery of the blood flow in the ischemic lower extremities causes the complex pathophysiological, biochemical and morphological processes [1,2] induced by 2 mutually dependent, but different pathophysiological mechanisms: "no-reflow phenomenon" and reflow-associated injuries called a "reflow-paradox" [3-5]. Reactive oxygen species (ROS) are the mediators of reflow-paradox in ischemia-reperfusion [2]. Lipid peroxidation (LPO) products cause both direct and indirect toxic effects on the vascular endothelium [6,7]. However, healthy people have a relatively low content of LPO products due to the presence of the regulatory systems that efficiently maintain the level of LPO products and inhibit LPO reactions when the oxidative processes are enhanced, thereby forming the body prooxidant-antioxidant balance [8,9]. The antioxidant system (AOS) that includes the enzymatic and non-enzymatic mechanisms for LPO product inactivation is a part of such protection [10].

The pathogenetic mechanisms of prooxidant-antioxidant state disorder with the reperfusion-reoxygenation syndrome (RRS) in reconstructive surgery of acute arterial failure in the lower extremities are not completely understood [7,11]. Acute muscular ischemia in the lower extremities was shown to be associated with tissue accumulation of LPO products [5]. LPO activation increases with the duration of ischemia and depends on the ischemic sensitivity of the organ [12]. The significant rise of malondialdehyde (MDA) and conjugated dienes (CD) in venous outflow from the ischemic leg was observed since 1-2 hrs after the start of reperfusion and was accompanied by increased edema [13,14]. In contrast, Yokayama K. et al. [15] showed somewhat later LPO activation: within the first 24-72 hrs after the operation.

Experiments in animals and clinical practice have shown that acute muscular ischemia in the lower extremities was associated with the reduction of plasma antioxidant activity [2]. The recovery of arterial circulation in the ischemized lower extremities caused further disorder of the AOS [10]. However, other investigators [12] did not find the differences in antioxidant enzyme activities (superoxide dismutase – SOD, glutathione peroxidase) in m.gastrocnemius between control and ischemized extremities after the reconstructive surgery of the arterial femoral-popliteal-tibial segment.

Therefore, the present investigation is aimed to study the role of the main parameters of prooxidant-antioxidant state in the development of the RRS during the surgical treatment of

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chronic leg ischemia caused by the atherosclerotic injuries of the femoral-popliteal-tibial arteries.

Material and methods

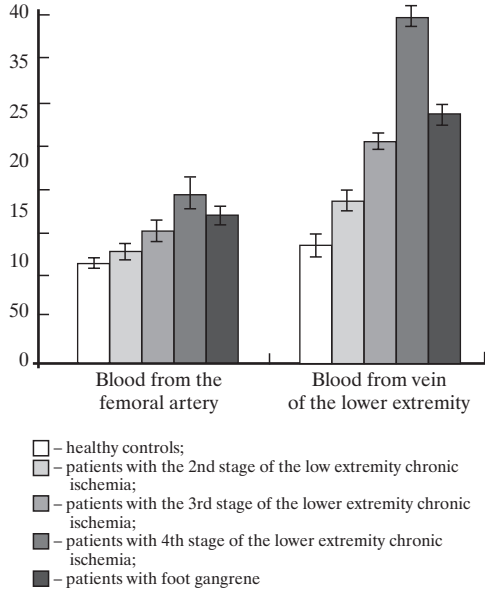
The study was carried out in 145 patients with atherosclerotic injury of the arteries of femoral-popliteal-tibial segment. According to the rules of the Grodno State Medical University Ethical Commission, the patients signed the informed consent for the blood sampling. 45 patients had chronic leg ischemia (CLI) in the lower extremities, stage 2 (by Fontaine), 61 patients had stage 3, and 28 patients – stage 4. Foot gangrene was diagnosed in 11 patients. Femoropopliteal autovenous bypass was performed in 122 patients. Profundoplastics was carried out in 12 cases. The reconstructive arterial operations in the lower extremities were performed under peridural anesthesia. The control group consisted of 34 patients without the signs of atherosclerotic arterial injuries in the lower extremities. 134 patients received usual drugs before and after the operations: vasodilator (xanthinol nicotinate) and disaggregant (pentoxyfilin).

The indices of prooxidant-antioxidant state were studied in plasma from the subcutaneous dorsal foot vein and from the common femoral artery of the ischemic leg. Blood was sampled before and on the 6th and 11th days after the reconstructive operations in the lower extremity arteries, and much later (1-5 years after the surgical intervention). LPO activity was estimated by CD and Schiff base (SB) concentrations. The CD content was measured by intensity of the UV absorbance at 232-234 nm (typical of lipid hydroperoxides with conjugated double bonds) [16]. SB level was determined by the fluorescence intensity of chloroform extract at excitation and emission wavelengths of 344 and 440 nm [16] measured with spectrofluorimeter "F-4010" (Hitachi). AOS state was evaluated by plasma content of α-tocopherol (α-TP) [17], β-carotene, coenzyme Q [18]. The data were processed by the routine methods of variation statistics using the software packages EXCEL and STATISTIC.

Results

The obtained results have shown that the development of CLI in the lower extremities because of the atherosclerotic occlusive or stenotic injury in the femoral-popliteal-tibial segment of the major arteries is associated with a significant ($p<0.01$) increase of CD and SB content vs the control group – both in arterial blood from the femoral arteries and in venous blood of the ischemic leg (*Fig. 1*). In addition, the progressing ischemic disorder in the lower extremities was accompanied by directly related activation of the free radical processes ($r=0.79$, $p<0.01$). The higher CLI stage was accompanied by more prominent accumulation of primary LPO products (CD vs SB). However, the persons with foot gangrene did not have further activation of the free radical processes comparing with patients with the 4th stage of the femoral lower extremity CLI; their CD and SB contents in the arterial and venous blood were even lower ($p<0.01$).

***Figure 1*. Schiff base (U/ml) in plasma from different vascular regions in patients with atherosclerotic injury of the femoral-popliteal-tibial arteries**



Simultaneously, the reduction ($p<0.01$) of both enzymatic and non-enzymatic AOS components was observed during the CLI progression (*Fig. 2*). The data of *Fig. 2* suggest that the degree of AOS reduction in our groups of patients also was directly related to the CLI stage ($r=0.86$, $p<0.01$). The lowest content of antioxidant was observed in patients with foot gangrene.

The recovery of the major artery blood flow through the femoral-popliteal-tibial segment of the patients that received routine therapy before and after the operation was accompanied by considerable LPO activation and AOS inhibition. Thus, *Fig. 1* shows that the highest CD and SB contents in the venous outflow from the reperfused-reoxygenated leg was observed on the 6th day after the operation; thereafter the levels of LPO products decreased, however, even 11 days after the operation it was significantly higher than before one ($p<0.01$). The activation of the free radical processes in the early postsurgical period directly depended on the initial CLI stage in the lower extremities ($r=0.88$, $p<0.01$). The relationship between the degree of LPO activation and the type of a reconstructive arterial operation (i.e. the volume of revascularized muscles) was observed. Thus, in the patients with the 3rd CLI stage in the lower extremities CD and SB content after profundoplastics was less than after bypass ($p<0.01$). Their highest CD and SB values were registered on the 11th day of the postsurgical observation.

The content of antioxidant continuously decreased within the first 6 days after successive bypass in the arterial femoral-popliteal-tibial segment (*Fig. 2*). Later the AOS state somewhat ameliorated with significant ($p<0.01$) increase in both enzymatic and non-enzymatic antioxidant levels comparing with their values on the 6th day. However, on the 11th day after the operation these levels were lower than before it ($p<0.01$).

In the cases of early shunt thrombosis within the first 11 days after the operation (10 cases) we did not note the previously

Figure 2. **Indices of antioxidant blood system from different vascular regions in patients with atherosclerotic injury of the arterial femoral-popliteal-tibial segment**

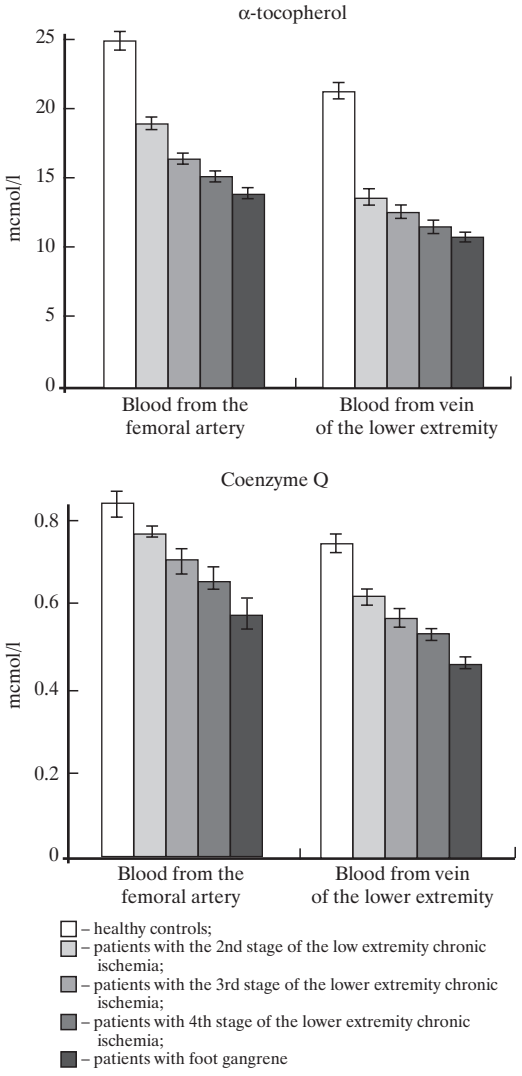
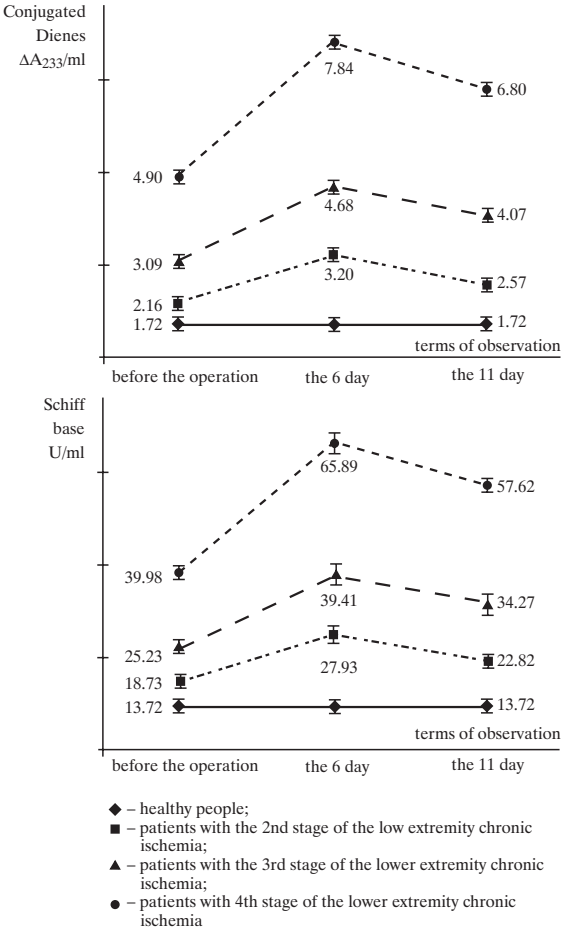


Figure 3. **Indices of lipid peroxidation in venous blood plasma from reperfused-reoxygenated the lower extremity in patients with atherosclerotic injury of the arterial femoral-popliteal--tibial segment**



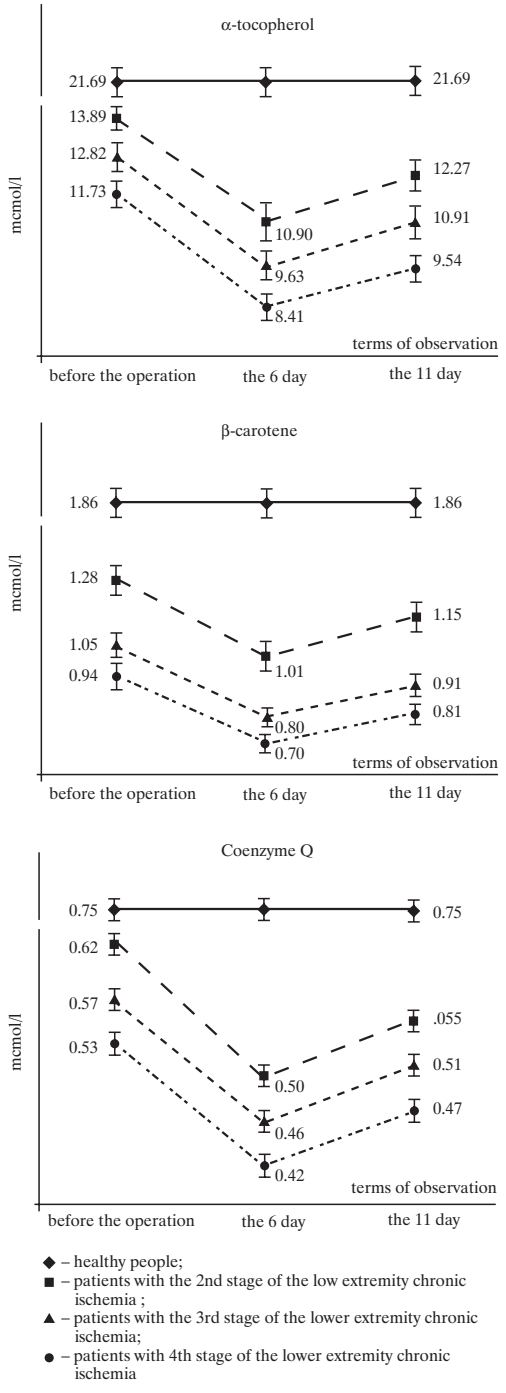
described dynamics of LPO activity and AOS state in blood obtained from the subcutaneous vein of the treated leg. The preoperative levels of LPO products and AOS indices in such patients were not changed after the operation. In persons with functional shunts the levels of CD and SB in the venous blood from the treated leg during the remote postsurgical period were essentially the same as in healthy controls, such as the values of AOS ($p>0.5$). In cases of later shunt thrombosis (12 observations) the values of investigated LPO and AOS parameters were similar to observed those in the patients with the 2nd (5), 3rd (4) or 4th (3) stage of the lower extremity CLI.

Discussion

The present investigation of LPO activity and AOS state during the atherosclerotic injury of the arterial femoral-popliteal-tibial segment indicated that chronic tissue hypoxia of the lower extremities was accompanied by the disorder of prooxidant-antioxidant balance. Such disorder was related to the activation of the free radical oxidative processes and AOS depletion and was directly dependent on the stage of CLI in the lower extremities. However, the development of irreversible trophic disorders in the ischemic legs diminished such dependence.

The return of the blood flow to the ischemized lower extremities of the patients with an atherosclerotic arterial injury in the femoral-popliteal-tibial segment is associated with an "oxygen burst" (dramatic increase of oxygen inflow) after the operation. Such burst initiates an active LPO process, expressed as a considerable rise of LPO products in the venous blood of the reperfused-reoxygenated leg. The degree of LPO activation is directly dependent on the CLI stage in the lower extremities, type and result of the arterial reconstruction performed. The observed increase in the lipoperoxide content is undoubtfully due to the beginning disparity between their generation and

***Figure 4*. Indices of antioxidant blood system from reperfused-reoxygenated the lower extremity in patients with atherosclerotic injury of the arterial femoral-popliteal-tibial segment**



insufficient physiological mechanisms limiting the LPO and AOS in the first turn.

The absence of excessive increase in venous LPO products in the reperfused leg after profundoplastics may be explained by lower blood flow to this leg. The multistep return of the blood flow to the ischemic tissues [19,20] may also have an important role in the slower peroxidative reactions in such patients. Ischemia is obviously associated with the imbalance between oxygen supply and velocity, efficiency of its cellular use. Furthermore, the efficiency of the oxygen consumption mechanisms, i.e. the relationship between the amount of generated free radicals and AOS activity is more important for LPO activation than absolute tissue oxygen content [9]. The close functional links between hemoglobin-oxygen affinity and as well activity of tissue free radical lipid oxidation were observed during hypoxia [9,21]. The direction and degree of changes in LPO processes and AOS activity in such conditions was shown to be dependent on the blood oxygen-binding properties [22]. These data about OA-induced LPO processes during the CLI in the lower extremities reveal a necessity to search antioxidant drugs for OA treatment.

Thus, the CLI development and progress in the lower extremities due to the atherosclerotic injuries of the arterial femoral-popliteal-tibial segment were accompanied by a disordered prooxidant-antioxidant balance. In patients with obliterating atherosclerosis such balance is shifted to the activation of free radical oxidation and inhibition of AOS. The recovery of arterial circulation in the ischemic lower extremities initiates the LPO activation, that depends on the blood flow to reperfused-reoxygenated leg, efficiency of oxygen use mechanisms and relationship between the amount of the generated radicals and AOS activity.

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Molecular mechanisms of brain plasticity: neurophysiologic and neuroimaging studies in the developing patients

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Abstract

The term plasticity, derived from the Greek word "plaistikos" meaning "to form" refers to the brain's ability to learn, remember and forget as well as its capacity to reorganize and recover from injury. There are four major types of plasticity: adaptive plasticity, impaired plasticity, excessive plasticity, and the 'Achilles heel' of the developing brain. Mechanisms of plasticity include: a change in the balance of excitation and inhibition; a long-term potentiation (LTP) or long-term depression (LTD); a change in neuronal membrane excitability; the anatomical changes-formation of new axon terminals and new synapses. Mechanisms for plasticity include activity-dependent refinement of neuronal connections and synaptic plasticity as a substrate for learning and memory. The molecular mechanisms for these processes were described in view of the current investigations. Authors presented: the role of calcium ions, calcium channels, NMDA receptors, free radicals, lipid peroxides and neurotrophins in the plasticity of developing brain. The utility of the neurophysiologic and MRI techniques were described in the determination of brain reorganization and repair in patients with cerebral palsy. Authors discussed their results on quntatative EEG and spectroscopy MRI studies in children with cerebral palsy. They have shown the existence of two processes in brain: brain damage and recovery.

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Introduction

The term plasticity, derived from the Greek word "plaistikos" meaning "to form" refers to the brain's ability to learn, remember and forget as well as its capacity to reorganize and recover from injury [1]. Children have enhanced capacity for learning and memory compared to adults as reflected in their ability to learn a second language, play musical instruments or become proficient in complicated sports such as golf or tennis. Children also have remarkable ability to recover from early brain injuries as demonstrated by their ability to recover receptive language after left hemispherectomy performed for epilepsy as late as the second decade [2]. Mechanisms of plasticity include: first, a change in the balance of excitation and inhibition; second, a long-term potentiation (LTP) or long-term depression (LTD); third, a change in neuronal membrane excitability; fourth, the anatomical changes, which need a longer period of time. Specific anatomical changes include formation of new axon terminals and new synapses.

In the 1980s and 1990s, a great deal of excitement was generated by new insights into new novel mechanisms of brain damage during hypoxia/ischemia [3,4]. It has been well known that hypoxia/ischemia lasting more than a few minutes can cause irreversible brain damage. Further research has indicated that reperfusion may cause more damage than simple hypoxia [4-6]. The mechanism of reperfusion injury is thought to involve the production of free oxygen radicals. The free radicals induce a chain reaction leading to a breakdown of the neuronal cell membrane (necrotic cell death). Further, free radicals are generated, causing a damage in the original cell that spreads to neighboring cells [5,6]. The brain uses glucose as its primary energy source. Glutamic acid, or glutamate, is a common metabolite of glucose metabolism. Glutamate is involved in several metabolic processes in the brain. It plays a role as a precursor for the inhibitory neurotransmitter, γ-amino butyric

acid (GABA). Elevated levels of glutamate were associated with increased brain activity. Furthermore, glutamate-induced excitotoxicity is a major mechanism by which neuronal loss may occur [6,7].

This review describes molecular mechanisms of brain plasticity and neurophysiological and magnetic resonance imaging studies in children with cerebral palsy.

Role of calcium ions (Ca^{2+})

Ca^{2+} is a regulator of metabolic pathways and serves important functions as a second messenger, thus the free cytosolic Ca^{2+} concentration must be tightly regulated at round 10^{-7}M. Ca^{2+} influx occurs by voltage dependent calcium channels (VDCCs) and receptor-operated calcium channels (ROCCs), while release form the endoplasmatic reticulum is triggered by inositol triphosphate [4,5,8]. Efflux of Ca^{2+} occurs by a high affinity-low capacity ATPase (calmodulin-dependent) and by a low affinity-high capacity, electrogenic Na^{+}/Ca^{2+} exchanger. Ca^{2+}-binding proteins buffer any Ca^{2+} entering the cell, or release within the cell. When Ca^{2+} concentrations increase, mitochondria become important in calcium storage. The Ca^{2+} sequestration process is dependent on ATP and during ischemia Ca^{2+} concentration within the cell rises [6,8,9].

The channels may be opened by the occupation of associated receptors (ROCCs) or by changes in membrane potential including those occurring with depolarization (VDCCs). The L-type Ca^{2+} channel is composed of five different polypeptide subunits, each with different molecular masses. The existence of this channel type has been demonstrated in many regions of the central nervous system (CNS) such as the cortex, hippocampus, cerebellum, and spinal cord.

The N (high-threshold inactivating) and T (low) type Ca^{2+} currents have also been documented. The T-type current could contribute to rhythmic firing of veretebrate neurons and the N or L-type currents could be involved in the release of neurotransmitters [10,12].

In general, it is thought that the blockade of T-type Ca^{2+} channels is associated with efficacy in treating absence seizures, while blockage of high voltage-activated (L-type) Ca^{2+} channels seems to be associated with control of partial seizures with or without secondary generalization [11-13]. More importantly, elevated levels of intracellular Ca^{2+} are thought to activate numerous Ca^{2+}-dependent processes that lead to cell death. Blockage of Ca^{2+} channels may play a key role in preventing these progressions [3,10,12].

L-type Ca^{2+} channels are expressed by particular cell types in the spinal sensorimotor network. They provide distinct non-linear conversions of synaptic input to axonal output. Several features suggest a major role in spinal motor function. The unusually slow kinetics is well adapted to provide the driving potential for the firing patterns that regulate the muscle activity of posture and locomotion. This arguably reduces the computational load on the premotor network [14]. Motor behaviour is the concerted action of hundreds of muscles. The study of postsynaptic properties mediated by L-type Ca^{2+} channels in spinal neurons has revealed mechanisms that may provide functional plasticity on a time scale from hundreds of milliseconds to tens of seconds [15].

Long-lasting changes in the structure and function of synapses occur in response to environmental stimuli in many regions of the nervous system during child and adult life.

The synaptic plasticity is a process called long-term potentiation (LTP) that is believed to be a cellular mechanism of learning and memory [16]. LTP is defined as a long-term enhancement of synaptic strength resulting from repeated activation of that synapse. In several regions of the brain, including the hippocampus, LTP has been shown to require activation of glutamate receptors and calcium influx into the dendrite of the post-synaptic neuron. Evidence also suggests that calcium release from endoplasmatic reticulum (ER) stores can promote LTP. And LTP can be modified by changes in ATP production and release. During LTP, mitochondrial calcium pump activity increases [17] and changes in mitochondrial gene expression occur [18]. Current findings suggest that such changes in mitochondria play an important role in the process of LTP. Calcineurin (CaN) exerts a powerful effect on a variety of signaling-proteins within neurons. Depending upon the strength, duration, and site of a Ca^{2+} stimulus, CaN may either increase or decrease synaptic efficacy and cell excitability, through modulation of ion channels, neurotransmitter receptors, cytoskeletal proteins, kinases, other phosphatases, and transcription factors. Thus, in response to multiple forms of synaptic stimulation, CaN initiates both short- and long-term changes on neuronal function, which ultimately translate to behavioral modification and neuroplasticity [17].

Role of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors

Developing neuronal connections are shaped by the balance of excitatory and inhibitory pathways entering the brain from primary sensory modalities such as vision, hearing and somatosensory sensation as well as by the activity of intrinsic circuits [16,18]. Most of these pathways use glutamate as their neurotransmitter, and active pathways are likely to gain influence compared with quieter ones according to their pattern of activation of glutamate receptors. Over-production of synapses during the postnatal period results in the 2 year old toddler having twice as many synapses in cerebral cortex as adults, and excessive synapses are pruned until approximately 16 years of age [19]. During this period, synaptic connections are refined through activity at excitatory synapses that use glutamate as their neurotransmitter [20]. Activity at glutamate synapses contributes to the loss of many synapses and the preservation of synapses that fire together repeatedly [21]. Both NMDA (N-methyl-D-aspartate) and AMPA (a-amino-3-hydroxy--5-methyl-4-isoxazole propionate)-type glutamate receptor activation is involved in synapse formation and stabilization. The NMDA-type glutamate receptor plays a special role in this process because it requires simultaneous stimulation by glutamate and membrane depolarization caused by stimulation of adjacent excitatory receptors [21]. Persistent coincident

firing of neurons leads to Ca^{2+} entry through NMDA receptors into post-synaptic neurons and release of trophic factors that support that synaptic connections [22]. Enhanced function of the immature NMDA receptor during postnatal development contributes to enhanced plasticity at this time [19,21,23]. Activation of NMDA channels allows Ca^{2+} to flux into neurons, activating protein kinases such as CaMKinase II and IV and production of neurotrophins such as brain-derived neurotrophic factor (BDNF). The growth factors such as BDNF, acetylcholine and serotonin receptors, and neurotransmitters that stimulate adenyl cyclase and protein kinase A all play roles in activation of gene transcription involved in neuronal plasticity [23,24]. Influx of Ca^{2+} through NMDA receptors plays an important role in LTP, formation of memories, and plasticity of neuronal circuits through downstream phosphorylation of transcription factors such as CREB (cyclic AMP response element binding protein transcription factor) as well as through indirect activation of transcription through the Ras-MAP kinase cascade [23].

AMPA receptor expression is also linked to synaptic morphology [24,28]. Synapses smaller than 180 nm across have been found to contain only NMDA receptors; above this size, the number of immunohistochemically detectable AMPA receptors increases linearly with synaptic diameter [29]. Increased AMPA receptor numbers are also associated with synaptic plasticity.

In the fetus or premature infant, expression of non-NMDA type glutamate receptors on immature oligodendroglia, during a critical window in development, makes them vulnerable to glutamate-mediated cell death leading to periventricular leukomalacia (PVL) [24]. Later in gestation, the enhanced function of immature NMDA receptors contributes to the selective vulnerability of neuronal circuits in the thalamus, basal ganglia and cerebral cortex to near-total asphyxia.

Free radicals (FR) and lipid peroxides (LP)

Free radicals (FR) and lipid peroxides (LP) are the byproducts of cellular metabolism that have been implicated in neurodegeneration, age-associated cognitive, memory impairments, ischemia and epilepsy [6,9,25-27,29]. Thus far, FR and LP have been treated as harmful agents that cause damage to macromolecules through nucleophillic attack [25]. However, there are recent indications that FR and LP may have an important role as signaling molecules that are used by cells as second messengers for altering the redox state of specific molecules that affect their functions [27,28]. These regulatory roles can only occur under physiologically significant concentrations of FR, which are strictly regulated in vivo by a myriad of antioxidant molecules. Recently, a link between FR and modulation of synaptic plasticity has been proposed; high concentrations of FR attenuate synaptic transmission and LTP [29]. On the other hand, superoxide radicals are proposed to be involved in LTP induction [30].

Nitric oxide (NO) is involved in the pathophysiology of brain ischaemia as well as in the formation of activity dependent synaptic plasticity. Accordingly, inhibition of nitric oxide synthase (NOS) attenuates anoxic LTP in the brain and this effect can be blocked by L-arginine (a substrate of NOS) [16].

PVL in the premature infant is a distinctive lesion of cerebral white matter associated with much of this adverse neurologic outcome. The pathogenesis of cerebral white matter injury in the premature infant is not entirely clear, although ischaemia-reperfusion and infection/inflammation appear to be important [31]. The unique nature of this neuropathological lesion in the premature infant is to relate in part to an exquisite vulnerability of the immature oligodendrocyte to FR and LP damages [31]. In two model systems of free radical accumulation, the early differentiating oligodendrocyte has been shown to be exquisitely vulnerable to FR attack [31]. Moreover, this immature form, the so-called preoligodendrocyte, has been shown recently to account for 90% of the total population of oligodendrocytes in cerebral white matter of infants under the gestational age of 31 week [32], the period when the premature infant is most at risk for white matter injury.

Neurotrophins

Neurotrophins belong to a family of secretory proteins that include nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3), and NT-4/5. Neurotrophins promote neuronal survival and differentiation, but it has become increasingly clear that they also have essential roles in neuronal survival and synaptic plasticity [22,33]. The expression of BDNF mRNA and the secretion of BDNF protein are tightly regulated by neuronal activity. Exogenous BDNF enhances transmission at the developing neuromuscular junction and at various central excitatory synapses [34]. Furthermore, endogenous BDNF, supports the survival, the growth of dendrites and axons, including glutamatergic neurons [35,36]. At cellular levels, the expression of BDNF mRNA is enhanced when the non-NMDA-type glutamate receptor is activated [37,38] and suppressed when GABA-A receptor is activated [39]. Cholinergic afferent inputs to the cortex and hippocampus also increase the levels of BDNF mRNA [37]. An important finding was that the enhancement of BDNF gene expression requires an increase in intracellular calcium concentrations [36] possibly by Ca^{2+} influx through L-type Ca^{2+} channels or NMDA receptors [38,39].

The local and synapse-specific modulation, together with preference in active neurons/synapses, suggests that neurotrophins must preferentially regulate active synapses with little or no effect on nearby less active synapses [37]. Moreover, both BDNF and NT-4 regulate cortical development. Specifically, exogenous application of BDNF regulates pyramidal neuron dendritic growth [40], whereas endogenous BDNF is necessary for differentiation of cortical interneurons [41].

Neurogenesis

Mechanisms that account for enhanced brain plasticity during childhood include persistence of neurogenesis in certain parts of the brain during the postnatal period, deletion of neurons through apoptosis or programmed cell death, proliferation and pruning of synapses, and activity-dependent

refinement of synaptic connections [3]. The presence of primitive ipsilateral non-pyramidal pathways can also contribute to plasticity as occurs in recovery of walking and body steering activity after injury to one hemisphere [4]. If damage to the corticomotoneuronal system occurs in adulthood, great difficulty follows in learning new or relearning former sequences of skilled movements [5], emphasizing the role of the corticomotoneuronal system in the acquisition and maintenance of skill. When lesions occur in the perinatal period, not only is learning of skilled movements severely impaired, but the development of alpha-motor neurons and their afferent segmental reflex control is secondarily disrupted [6,7]. This implies a further role for the corticomotoneuronal system in man: activity dependent regulation of the development of spinal motor centres.

In animals, it has been demonstrated that exercise increases the number of new neurons [49]. Neurotrophins might mediate this effect. Exercise increases levels of BDNF in the hippocampus and BDNF promotes the survival of newly differentiated neurons. Exercise increases brain uptake of circulating IGF-1, a factor that promotes neuronal differentiation of progenitor cells and increases hippocampal BDNF gene expression [49,50]. In addition, Fibroblast growth factors (FGF-2) which stimulates proliferation and differentiation of hippocampal cells, and there is increased in hippocampal astrocytes after exercise [51]. Thus, exercise activates a number of factors that converge on neurogenesis.

There are four major types of plasticity: adaptive plasticity, impaired plasticity, excessive plasticity, and the 'Achilles heel' of the developing brain [42]. Adaptive plasticity refers to the changes in neuronal circuitry that enhance a special skill with practice or allow the brain to adapt or compensate for injuries or changes in sensory input. Impaired plasticity refers to situations in which genetic or acquired disorders disrupt molecular plasticity pathways (genetic and acquired disorders that cause cognitive impairment). In contrast, excessive plasticity in the developing brain can lead to disability through reorganization of new, maladaptive neuronal circuits that cause neurologic disorders such as partial seizures following mesial temporal sclerosis or focal dystonia. Plasticity becomes the brain's 'Achilles heel' in situations such as energy failure or status epilepticus when excitatory mechanisms designed to enable plasticity become over-stimulated, resulting in excitotoxic neuronal damage.

More recent studies have concentrated on the recovery and plasticity in the stroke patients [43-46]. A small number of the investigations have been performed on children with cerebral palsy (CP) [47,48].

Cerebral palsy

The CP prevalence is increasing, more premature infants survive because of better neonatal care [52]. Spastic diplegia is the commonest form of CP as a results of injury to the PVL, which occurs only during a temporal window of development that ends at 30-32 weeks PCA. A characteristic feature of PVL is disruption of corticospinal axons, while the cortical pyramidal projection neurons are left intact and subsequently make aberrant intracortical axonal projections [53]. The rapidly expanding understanding of CNS axonal regeneration indicates that with early intervention there are realistic prospects of inducing corticospinal axons to re-grow through the cystic areas of PVL and to find their appropriate targets [54]. Myelin is inhibitory to axonal growth but this should not pose an encumbrance to axonal regrowth, since the corticospinal tract is poorly myelinated before term [55,56]. Recently, it has been demonstrated that corticospinal axons are actively growing, innervating spinal cord and expressing growth-associated protein-43 (GAP43) during this period and are thus likely to have a high degree of plasticity. Interventions providing early regeneration of corticospinal projections and reinnervation of the spinal cord in preterm babies with PVL would be likely to reduce disability, not only by re-establishing the cortical input to spinal motor centres but also by facilitating their subsequent normal development.

There are a few tools to study brain plasticity. Recently, neural plasticity can be measure evaluated validly with transcranial magnetic stimulation techniques (TMS), mapping EEG and functional magnetic resonance imagines (fMRI).

EMG studies

Carr and colleagues [57] studied the central motor reorganization in subjects with hemiplegic CP. The cortico-spinal projections were investigated using focal magnetic stimulation of the motor cortex. Reflex pathways were examined with digital nerve stimulation. In 64% of the patients, there was evidence for reorganization of central motor pathways. The clinical and neurophysiological findings revealed two different forms of reorganization. In both forms focal magnetic stimulation demonstrated novel ipsilateral motor pathways from the undamaged motor cortex to the hemiplegic hand. Ipsilateral projections were not demonstrated from the damaged motor cortex. In these subjects cross-correlation analysis and reflex testing suggested that corticospinal axons had branched abnormally and projected bilaterally to homologous motor neuron pools on both sides of the spinal cord. It was demonstrated that good function of the hemiplegic hand was associated with the presence of EMG responses in that hand following magnetic stimulation of the contralateral motor cortex. When EMG responses were absent, hand function was poor unless the subject had intense mirror movements.

Transcranial magnetic stimulation (TMS), functional MRI (fMRI) studies

In the motor an sensory system, TMS studies demonstrated cortical plasticity in CP patients [58-60]. Maegaki and colleagues [58] investigated central motor reorganization for the arm and leg muscles in spastic and athetoid CP patients with bilateral cerebral lesions using TMS. On computer tomography and MRI, bilateral PVL were observed in all spastic patients with preterm birth. Ipsilateral responses were more common among CP patients, especially in TMS of the less damaged hemisphere

in patients with marked asymmetries in brain damage. The cortical mapping of the sites of highest excitability demonstrated that the abductor pollicis brevis and biceps brachii sites in CP patients were nearly identical to those of the normal subjects. These results suggest that ipsilateral motor pathways were reinforced in both spastic and athetoid CP patients, and that a lateral shift of the motor cortical area for the leg muscle may occur in spastic CP patients with preterm birth.

Thickbroom et al. [59] used TMS and fMRI to investigate cortical motor and sensory areas in children with hemiplegic CP. Both TMS and fMRI demonstrated a normal contralateral motor and sensory projection between the unaffected hand and the cerebral hemisphere. However, in the case of the affected hand, the TMS results indicated either a purely ipsilateral projection or a bilateral projection in which the ipsilateral pathway had the lower motor threshold, whereas passive movement resulted in fMRI activation in the contralateral hemisphere. A significant fast-conducting corticomotor projection to the affected hand from the ipsilateral hemisphere was demonstrated. Also, the predominant afferent projection from the hand was directed to the affected contralateral hemisphere, resulting in an interhemispheric dissociation between afferent kinesthetic inputs and efferent corticomotor output. The authors [59] have concluded that there are differences in the organization of sensory and motor pathways in CP. They have also suggested that the motor dysfunction experienced by these subjects could be due to an impairment of sensorimotor integration at cortical level as a result of reorganization in the motor system.

In another study, Staudt and colleagues [60] have evaluated the impact of different lesion extents on the type of reorganization induced in young adult patients with congenital hemiparesis. The severity of structural damage to hand motor projections of the corticospinal tract was assessed on semi-coronal MRI reconstructions along anatomical landmarks of corticospinal tract somatotopy. The functional integrity of these crossed corticospinal projections in the affected hemisphere, as well as the presence of any abnormal ipsilateral projections to the paretic hand, was examined by TMS. The cortical activation during simple voluntary hand movements was studied by functional MRI (fMRI). Patients with small lesions and only mild hand motor impairment possessed intact crossed corticospinal projections to the paretic hand, whereas no motor response could be elicited by TMS of the affected hemisphere in those with large lesions and more severe hand motor impairment. Evidence for compensatory recruitment of the unaffected hemisphere was found in both subgroups. In the small lesion group, fMRI demonstrated ipsilateral activation of premotor areas, without any abnormal projections to the paretic hand originating from these sites. In the large lesion group, such abnormal ipsilateral projections to the paretic hand were indeed found, and fMRI confirmed cortical activation of an abnormal ipsilateral hand motor representation in the primary sensorimotor region of the unaffected hemisphere. They concluded [60] that the type of corticospinal reorganization depends on the extent of the brain lesion.

Coherence EEG studies

The EEG changes in CP patients generally reported are non-specific [61,62]. On the other hand a quantitative EEG (power spectra and coherence) provides objective measures in the search for global or focal abnormality which, if present, may signal an underlying organic process [63,64]. The coherence is function of frequency [65]. Coherence is an amplitude independent measure of phase synchrony between EEG signals, reflecting functional interregional coupling and depending mainly on structural connections [64,65]. The coherence values are interpreted in terms of various connectivity between brain structures [66]. Coherence has been found to vary with numerous diseases states. Certain regions and frequency range increases in coherence in multi-infarct dementia, AIDS and mild head injury, while it decreases in Alzheimer's disease and depression [66-68].

Koeda et al. [69] have evaluated EEG spectral power density, interhemispheric (ICoh) and intrahemispheric (HCoh) coherence, and asymmetry of coherence between the right and left hemispheres in twelve children with spastic diplegia (SD). No significant differences in EEG spectral power density were observed in these patients. The authors noted lower ICoh at the occipital pair for the alpha band and a higher value at the frontal pair for the theta band in SD children. Higher HCoh in SD was pronounced in the left hemisphere for the delta, theta, and beta bands. On the other hand, there were no higher values in the control group. Higher HCoh asymmetry was exhibited in the left hemisphere in the control group, while very little asymmetry was found in the SD group. We suggest that these neurophysiologic abnormalities in preterm SD children correspond neuroanatomically to the callosal thinning and neuropsychologically to the visuoperceptual impairments. These findings are agreement with our results [70]. In our previous report on MRI findings in CP patients, we have observed callosal thinning more expressed in the tetraplegic patients than in SD ones [70].

We have studied the quantatative and coherence EEG on the lager group of patients with spastic diplegia [71]. A group of twenty-nine children with SD was studied. EEG records were compared to healthy children with normal EEGs. For every subject, twenty artifact-free EEG epochs, each of 2 s duration, were selected for spectral analysis and coherence functions. Significant decrease of power alpha at occipital derivations was demonstrated in children with SD in comparison to the control group. On the other hand, there was an increase in theta power and delta bands almost in all leads. Significant decrease of ICoh coherence values in children with SD for the alpha and delta bands in frontal and central leads, as compared with controls, were observed (*Fig. 4*). However, higher ICoh coherence values were detected at frontal, central, parietal and occipital leads for the alpha, theta and beta1 bands. Lower HCoh coherence values in the patients at temporal-occipital derivations were noted. In contrast, we have also detected higher HCoh values at temporal and temporal-occipital derivations for delta and beta bands. The presented results support anatomic-neurophysiologic abnormalities and the existence of compensatory mechanisms in children with SD.

Figure 1. **Patient aged 7, spastic diplegia, hyperintensity changes in thalamus (white arrows) (MRI-T2). (Kułak et al., 2004 [70])**

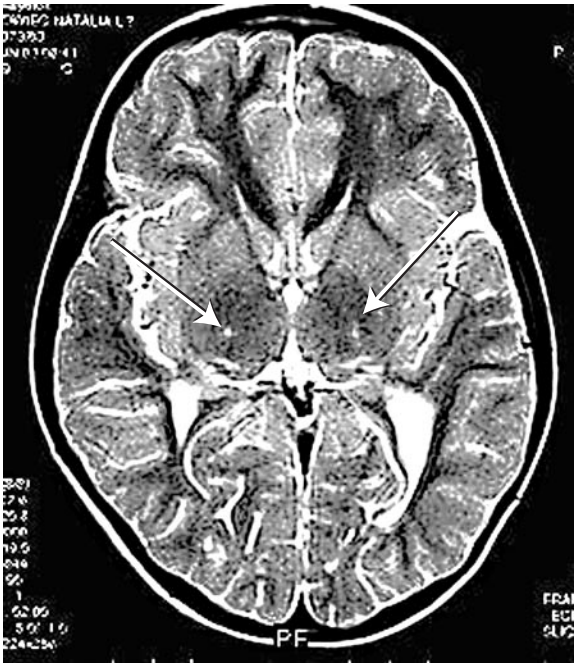


Figure 2. **Patient aged 11, with spastic CP tetraplegia. Paraventricular leukomalacia with hyperintensive changes in frontal and parietal lobes – black arrows, with wide sulci of cortex and enlargement of ventriculi (MRI-FLAIR). (Kułak et al., 2004 [70])**

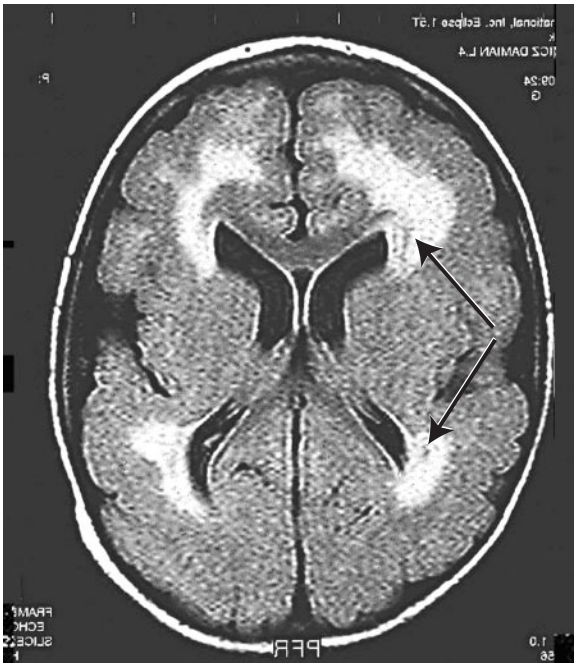
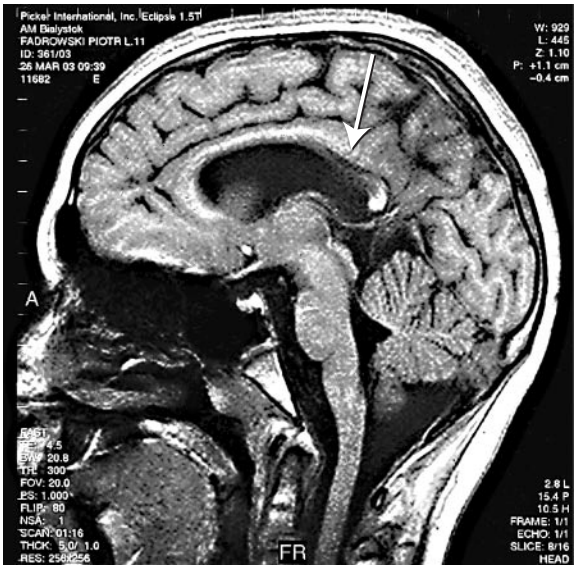


Figure 3. **Patient aged 11, with spastic CP tetraplegia. Thinning of corpus callosum – white arrow. Wide sulci of cortex and enlargement of ventriculi (MRI-T2).(Kułak et al., 2004 [70])**



Hemiparetic cerebral palsy (HCP) is a form of CP. HCP often predicts which patients will develop cognitive disabilities and/or unprovoked seizures. Children whose hemiparesis involves the upper limb to a greater extent than the lower one (arm-dominant hemiparesis) are much more likely to experience learning difficulties than those whose clinical pattern is leg-dominant. Patients with arm-dominant hemiparesis tend to have relatively large lesions involving cortex and subcortical white matter (e.g. major arterial territory infarcts, porencephaly, schizencephaly, polymicrogyria, cortical and subcortical atrophy) and would therefore be more likely to develop learning difficulties and epilepsy.

We have analyzed the spectral and coherence EEG in children with spastic hemiplegia [72]. A group of fourteen children with right hemiparetic cerebral palsy (RHCP), ranging from 6-14 years of age was studied. The second group consisted of twelve children with left hemiparetic cerebral palsy (LHCP) of similar age. In this study we have found significant differences in the distribution of the alpha, theta, delta and beta rhythm between HCP and control children over the left and right hemisphere. There were highly significant differences between the HCP and controls in the distribution of the theta rhythm over left hemisphere. The lower ICoh at the temporal, parietal and occipital derivations in the alpha band implies hypoconnectivity between the right and left hemispheres and suggests hemistructural brain lesion (*Fig. 5*). The HCoh asymmetry, which implies relative hypoconnectivity within the left hemisphere, as compared with the right one, suggests that functional hemispheric differentiation may be diminished. Our results suggest a possible increase in the plasticity of the brain in children with CP. We postulate that the rehabilitation efficacy of children with CP can be measured by EEG coherence.

***Figure 4*. Differences of interhemispheric (ICoh) and intrahemispheric (HCoh) in children with spastic diplegia (SD). Solid lines indicate significantly lower ICohs and HCoh in SD children compared to normal subjects. Dashes indicate significantly higher ICohs and HCoh in SD children compared to the control group (Kułak et al., 2003 [71])**

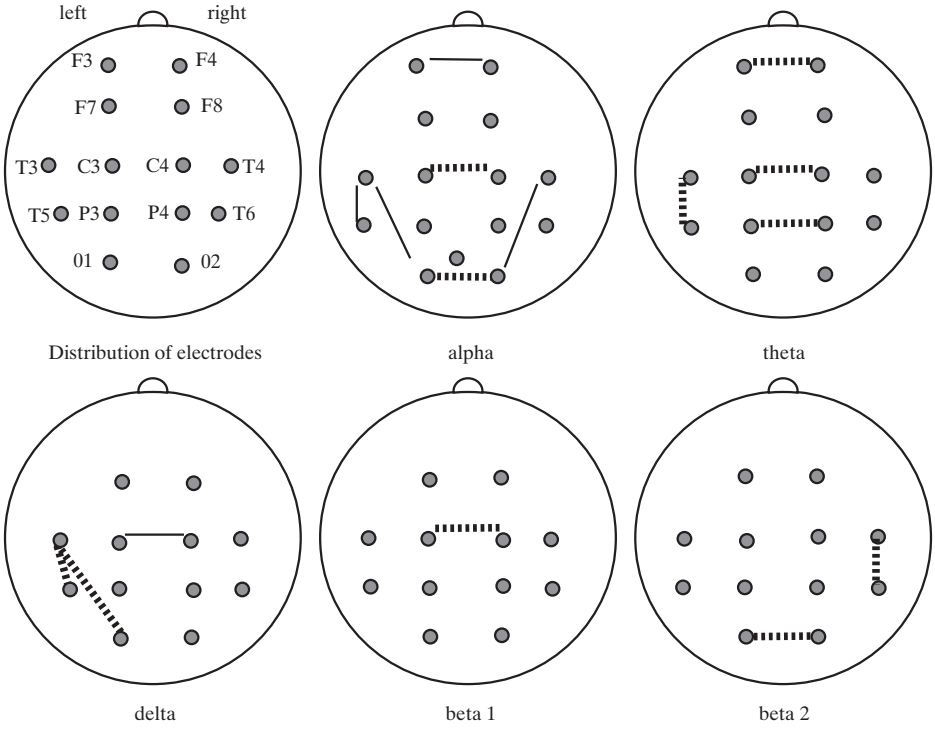


***Figure 5*. Differences of HCoh between right hemiparetic cerebral plasy (RHCP) children and the control group. Solid lines indicate significantly lower HCohs in HCP children compared to normal subjects. Dashes indicate significantly higher HCohs in RHCP children compared to the control group (Kułak et al., 2004 [72])**

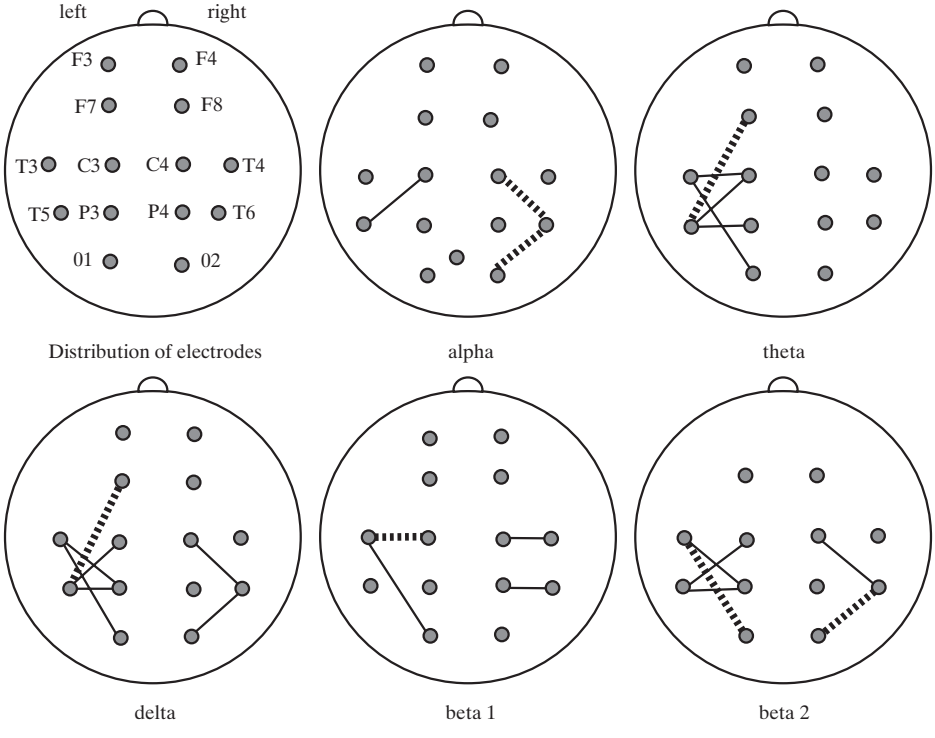


Table 1. **Metabolites ratios in the left basal ganglia in children with spastic diplegia (SD) and control group (X ± Standard Deviation) (Kułak et al., 2004, [77])**

Metabolites	SD group, n=19	Control group, n=19	t value	p value
NAA/Cr	1.63 ± 0.18	1.99 ± 0.16	t= 5.95	p<0.001
NAA/Cho	1.98± 0.26	2.17 ± 0.17	t=2.58	p<0.05
NAA/mI	3.08 ± 0.68	3.84 ± 0.70	t= 3.33	p<0.01
Cr/NAA	0.59 ± 0.06	0.49 ± 0.02	t=5.43	p<0.001
Cr/Cho	1.15 ± 0.13	1.06 ± 0.10	t= 2.11	p<0.05
Cr/mI	1.87 ± 0.38	1.92 ± 0.37	t=0.45	NS
Cho/NAA	0.51 ± 0.06	0.47 ± 0.04	t=2.28	p<0.05
Cho/Cr	0.87 ± 0.09	0.95 ± 0.10	t=2.31	p<0.05
Cho/mI	1.59 ±0.33	1.81 ± 0.27	t=2.17	p<0.05
mI/NAA	0.33 ± 0.07	0.26 + 0.04	t=3.55	p<0.01
mI/Cr	0.58 ± 0.13	0.53 ± 0.07	t=1.43	NS
mI/Cho	0.66 ± 0.66	0.57 ± 0.11	t=1.62	NS

two tailed t-test, NS – not significant; N-acetylaspartate (NAA), creatine (CR), choline (Cho), myo-inositol (mI)

MRI and MRS

Proton magnetic resonance spectroscopy (^{1}H MRS) is widely applied in the determination and differentiation of brain tumors, hypoxia, post radiotherapy changes and other lesions mimicking neoplasm-like abscesses [73,74]. ^{1}H MRS has proved to be a useful tool for the early evaluation of brain injury in asphyxiated neonates [75]. The basal ganglia, and more specifically the corpus striatum, play a central role in the feedback loop that modulates cerebral cortical function [76]. Disruption of corticostriatal pathways seems to be of particular importance with regard to neurobehavioral abnormalities. This is highly relevant in CP children because, for several reasons the basal ganglia are vulnerable to injury during a restricted period in brain development. High lactate (Lac) levels and low N-acetylaspartate (NAA) levels are the most common findings. A fall in the NAA/choline (Cho) ratio also indicates an adverse prognosis. NAA is a neuronal marker, the other metabolites can be viewed as cerebral indicators of energy metabolism creatine (Cr) and of cytoplasmic Lac and mitochondrial glutamate-glutamine (Glx) redox state. Myo-Inositol (mI) is a precursor for the phosphatidylinositol second messenger system, regulates osmotic processes within the brain and takes part in mood states. We used ^{1}HMRS to children with SD to determine the metabolite profile of SD children in the basal ganglia, and the relationship of this profile with motor and mental development [77]. ^{1}H-MR spectroscopy single-voxel (8 cm^3) located in the left basal ganglia (thalamus, capsula interna) from an axial section was applied. Seventeen SD children had hypoxic-ischaemic lesions with patterns of PVL (see *Fig. 1*, *2* and *3*) subcortical lesions or cortical infarction in MR. Two patients had normal MR scans. The reduced ratios of NAA/Cr, NAA/Cho, NAA/mI, Cho/NAA, Cho/Cr and Cho/mI of SD children and controls in the basal ganglia differed significantly (*Tab. 1*). On the other hand, we noted increased ratios of Cr/NAA, Cr/Cho and mI/NAA in the SD patients as compared with the controls. NAA/mI ratios were positively correlated with the severity scale of CP in SD children. Cr/NAA ratios were significantly correlated with the mental retardation in SD patients. In this study, we found reduced values of NAA and Cho in the basal ganglia in SD children. This may be indicative of neuronal loss subsequent to an anoxic episode during the prenatal or perinatal periods. After all, elevated Cr values in SD children may reflect an increase in the metabolism in the basal ganglia and the existence of compensatory mechanisms-plasticity. And increased mI/NAA ratios suggest the existence of gliosis in the examined patients. Only in one study [78] of Krägeloh-Mann et al., ^{1}H MRS was performed on two children with dyskinetic CP. They found a decrease in NAA/Cr and Cho/Cr ratios in the basal ganglia. To our knowledge, no HMRS study has been conducted in children with SD. We postulate that HMRS may be a useful technique in the determination of plasticity processes in the brain.

Conclusions

Children with cerebral palsy have remarkable ability to recover from early brain injuries. Mechanisms of brain plasticity include: a change in the balance of excitation and inhibition; a long-term potentiation or long-term depression; a change in neuronal membrane excitability; the anatomical changes, which need a longer period of time.

The molecular mechanisms of brain plasticity are under intensive researches. Calcium ions, calcium channels, NMDA receptors, free radicals, lipid peroxides and neurotrophins play a major role in these processes. At present, the neurophysiologic and MRI techniques are able to disclose the plasticity in children with cerebral palsy. Quntitative EEG and spectroscopy magnetic resonance are useful tools in the determination of plastic changes in children with cerebral palsy.

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Collagen type I and III metabolism in assessment of mandible fractures healing

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Abstract

Purpose: The aim of this work was estimation of the PIIINP, PICP and ICTP concentrations in blood serum during non-complicated mandible fracture healing; settlement of dependences between kinetics of changes of examined markers with reference to particular bone fracture phases and applied treatment methods; the determination of usefulness of collagen metabolism markers type III and I for the monitoring of mandibular fracture healing.

Material and methods: The material was blood serum of men aged 20-30 years, which were treated on mandible fractures in Maxillofacial Clinic Medical University of Białystok. Depending on the treating method, examined patients were divided into two groups. Patients treated with non-operative method were I group (n=31), II group was made of patients treated with surgery (n=33). The concentrations of markers measured on the 2nd, 14th, 42nd, 90th day after trauma and in II group these substances were measured additionally on the 2nd and 14th day after surgery. Control group consisted of 20 healthy men the same age. Concentrations of markers were measured with the radioimmunological method (RIA).

Results: Regular process of mandible fracture healing in men in various periods occurs with PICP, PIIINP and ICTP concentration changes in blood serum.

Conclusions: Changes of maker concentration show that, mandible fracture healing treated non-operatively is a more dynamic process than stable osteosynthesis method applied. Lack of positive correlation of the PIIINP and PICP concentration in blood serum of patients in two examinated groups can indicate on the different machanisms of mandible farcture healing connected with different methods of the treatment.

Key words: fracture healing, biochemical markers, PIIINP – N-terminal propeptide of type III procollagen, PICP – C-terminal propeptide of type I procollagen, ICTP – C-terminal telopeptide of type I collagen.

Introduction

The process of bone fracture healing can be assessed by using many present diagnostic methods [1-4]. However, they are of limited usefulness in clinical practice due to their invasive character or late manifestation of changes occurring during bone regeneration. Thus, there is a necessity for non-invasive and repetitive methods enabling assessment of fracture healing at early stages [5-8]. Taking into account the importance of collagen synthesis in the process of bone healing such requirements can be fulfilled by biochemical markers of collagen metabolism, i.e. collagen synthesis and degradation products released into the blood.

Experimental studies have shown that during fracture healing with immobilization of bone fragments, collagen type I and III are synthesized in variable quantitative and temporary relations [2,3,9]. In the estimation of type III collagen available for examination, the N-terminal propeptide of type III procollagen (PIIINP) is a marker of collagen III type metabolism, i.e. both its synthesis and degradation [10].

The C-terminal propeptide of type I procollagen (PICP) is a marker of type I collagen biosynthesis and also a marker of bone tissue formation. The propeptide is released during collagen synthesis from precursor molecule [5,8,11]. The C-terminal telopeptide of type I collagen ICTP which contains net binding,

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is a marker of bone resorption. The telopeptide is formed during degradation of mature fibres of type I collagen [5,8,11,12].

The markers are of non-invasive character, are repetitive and have the possibility of duplication of various biochemical determinations. The possibility of assessment of resorption and bone tissue formation, i.e. basic processes taking place in fracture healing, is their advantage.

The evaluation of fracture healing using markers of collagen type III and I have been a subject of single experimental [1] and clinical [13-18] studies. Clinical studies covered a small number of patients, diversified as far as the kind of fracture, methods of treatment, age and sex of patients were concerned.

Including above-mentioned data, a study on men with mandible fractures in various stages of regular healing was conducted. The aims of the study were:

– estimation of the PIIINP, PICP, and ICTP concentrations in blood serum during non-complicated mandible fracture healing

– settlement of dependences between kinetics of changes of examined markers with reference to particular bone fracture phases and applied treatment methods

– determination of usefulness of collagen metabolism markers type III and I for monitoring of mandible fracture healing.

Material and methods

The examined group comprised of 64 men, aged 20-30, treated in the Maxillofacial Clinic, Medical University of Białystok due to mandible fractures. The following criteria qualified patients for the study: mandible fracture diagnosed on the basis of clinical and radiological examinations; lack of skin wounds and other bodily injuries including fractures and the central nervous system damage; case history, physical examination and basic laboratory blood and urine tests did not show past or coexisting systemic and metabolic diseases; the patients were not treated for bone fractures; did not take hormones, anticoagulants, anticonvulsants, diuretics, calcium and magnesium preparations, vit. D prior the trauma, they were not alcohol- and narcotic-addicted; were not under the influence of alcohol on the day of accident; had clinically satisfactory state and paradontal tissues; gave a written consent to 4 or 6 times of blood collection for test in appropriate time intervals.

Depending on the method of treatment of mandible fracture, the patients were divided into 2 groups: I – the patients treated with non-operative method (n=31) and II – the patients treated operatively (n=33). Single mandible fractures were observed in 18 patients (58.1%) and multiple fractures – in 13 patients (41.9%) of the first group. The most frequent localization of mandible angle (36.1%), mandible body in the region of molar teeth (17%), and condyloid process (14.9%). Multiple fractures were presented in 22 patients (60.6%) and simple fractures in 11 patients (39.4%) of the second group. Bone injuries concerned mainly mandible angle (25%), condyloid process (21.1%), and the region of molars (17.3%).

Non-operative treatment was based on application of splints and elastic intermaxillary traction, which was changed into permanent fixation after bone fragments reduction. The period of intermaxillary immobilization was 6 weeks.

Mandible fractures were treated operatively in the II group. The procedure was conducted under general anesthesia with intratracheal intubation and the section was performed intraorally or extraorally. Bone fragments were repositioned and fixed using miniplates, which were fixed with screws. One-sided osteosynthesis was performed in 11 patients, both-sided – in 22. Additional intermaxillary immobilization stretched on the splints, to fix occlusion prior to the trauma, was applied in the patients of this group in the first 24 hours after the surgery. Screws and plates, used for osteosynthesis, were removed after 3 months.

The patients with mandible body fracture, due to contact of fracture fissure with oral environment and those treated surgically, received antibiotic for 5-7 days. Clinical and radiological evaluation revealed bone fracture healing without complications. During both hospitalization and ambulatory treatment, blood was taken in assigned periods to determine PIIINP, PICP, and ICTP; in groups I and II – on the 2nd, 14th, 42nd, and 90th days after the trauma and in the II group – moreover on the 2nd and 14th days after operation. The days of markers determination in the course of mandible fracture healing were settled on the basis of the duration of 4 healing phases (in the mechanism of spontaneous concrescence). They resembled the following phases of the process: the 2nd day – phase I (inflammatory), the 14th day – phase 2 (granulation), the 42nd day – phase 3 (callus, i.e. clinical concrescence), and the 90th day – phase 4 (callus reconstruction) [19-21]. The examinations on the 2nd and 14th days after operation were to establish the influence of the operation and soft tissue healing on these markers concentration. It should be noticed that the material taken on the 90th day after the trauma was collected before miniplates removal.

The blood was collected from the elbow vein, on empty stomach between 7 and 8 a.m. to the testing tubes to obtain clots. After clotting, the blood was centrifuged and serum were frozen at the temperature of -80^0C until metabolism markers determination of collagen types III and I was conducted. Similarly, the blood of healthy men (volunteers) aged 20-30, the control group, was collected between 7 and 8 a.m. on empty stomach.

Blood serum PIIINP, PICP and ICTP concentrations were determined with radioimmunological method, using ready RIA kits, Orion Diagnostica (Finland). The results were presented as arithmetic means for particular groups of men and defined periods of fracture healing with regard to standard deviation – SD. Statistical differences between the examined groups and the control group on consecutive days of healing, were conducted using Student-t test for unpaired trials. Unpaired t-test was used to compare the results of particular days of healing. Differences at the level of confidence $p<0.05$ were considered significant. In order to establish mutual relationships between PIIINP, PICP, and ICTP concentrations in the course of fracture healing, a correlation coefficient was calculated, by comparing mean concentration of consecutive days of fracture healing. The correlation was considered significant (positive or negative) coefficient r values exceeding 0.9.

Figure 1. **PIIINP concentrations in blood serum in I group of patients in examined days of healing and control group (K)**

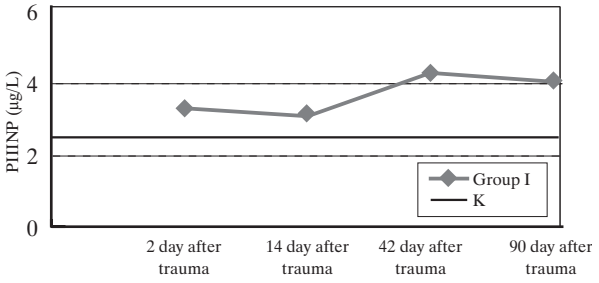

Figure 2. **PIIINP concentrations in blood serum in II group of patients in examined days of healing and control group (K)**

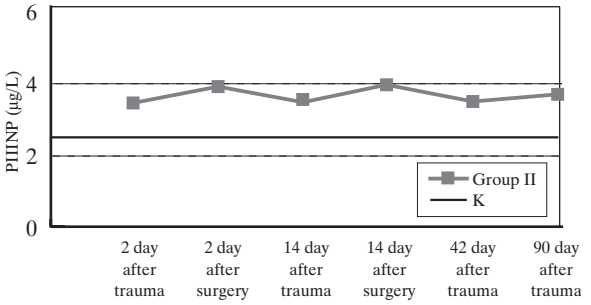

Table 1. **PIIINP concentrations in the blood serum in particular days of healing in I group of patients and control group**

The days of examination after trauma	2	14	42	90	Control group
PIIINP concentration (μg/L) ±SD	3.32 (±0.94)	3.10 (±0.93)	4.30 (±1.24)	4.09 (±1.21)	2.49 (±0.67)
Level of significance (p)	p<0.002	p<0.02	p<0.0001	p<0.0001	-

Table 2. **Statistical differences between PIIINP concentrations in particular days of healing after trauma in I group of patients (NS – statistically non-significant)**

The days of examination after trauma	2:14	2:42	2:90	14:42	14:90	42:90
Level of significance (p)	NS	p<0.001	p<0.01	p<0.0001	p<0.001	NS

Table 3. **PIIINP concentrations in the blood serum in particular days of healing in II group of patients and control group**

The days of examination	2nd day after trauma	2nd day after surgery	14th day after trauma	14th day after surgery	42nd day after trauma	90th day after trauma	Control group
PIIINP concentration (μg/L) ±SD	3.38 (±0.94)	3.83 (±1.09)	3.40 (±0.99)	3.88 (±1.16)	3.42 (±1.02)	3.62 (±1.02)	2.49 (±0.67)
Level of significance (p)	p<0.001	p<0.0001	p<0.001	p<0.0001	p<0.001	p<0.0001	-

The Senate Committee for Ethics and Supervision of Studies on People, Medical University of Białystok gave its consent for the study.

Results

Having done statistical calculation with the division of patients into subgroups with regard to simple and multiple mandible fractures, we did not observe any significant differences in connection with treatment method. Thus, the results were presented in two groups, group I – patients treated with non-operative method and group II – operative method.

PIIINP

Group I. The blood serum of patients treated with non-operative method in the course of mandible fracture healing showed the increase in PIIINP concentrations on all examination days as compared to the control group (K). On the 2nd and 14th days after the trauma, PIIINP concentrations were approximate. The highest concentration of propeptide was observed on the 42nd day after the trauma. As compared to this period, PIIINP concentrations in the last examination (the 90th day after the injury) were only slightly lower (*Fig. 1*).

Propeptide concentrations increase, observed in all periods of mandible fracture healing in patients treated with non-operative method were statistically significant as compared to PIIINP concentrations in group K (*Tab. 1*).

Statistically significant differences were obtained comparing PIIINP concentrations between days 2:42, 2:90, 14:42, 14:90 (*Tab. 2*).

Group II. The patients treated operatively showed, similarly to group I, PIIINP concentrations increase in all examination days of fracture healing as compared to group K. Propeptide concentrations on the 2nd, 14th, 42nd, and 90th days after the trauma were very close. The highest concentrations of PIIINP were observed on both days after operation (the 2nd and 14th). The concentrations were on similar levels (*Fig. 2*).

Propeptide concentrations increase, observed on particular examination days of mandible fracture healing, after the injury and after the operation, was statistically significant, as compared to PIIINP concentrations in group K (*Tab. 3*). There were no statistically significant differences comparing PIIINP concentrations between examination days after the trauma.

We did not find positive correlation comparing mean concentrations of PIIINP obtained on particular examination days after the injury in patients of both groups.

Figure 3. **PICP concentrations in blood serum in I group of patients in examined days of healing and control group (K)**

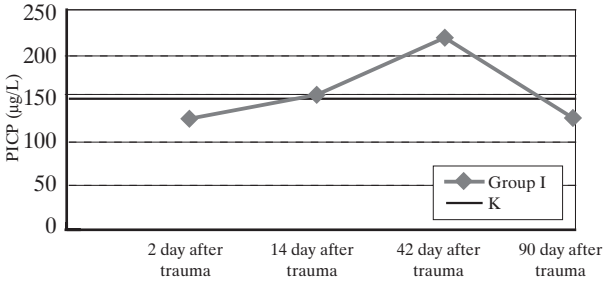

Figure 4. **PICP concentrations in blood serum in II group of patients in examined days of healing and control group (K)**

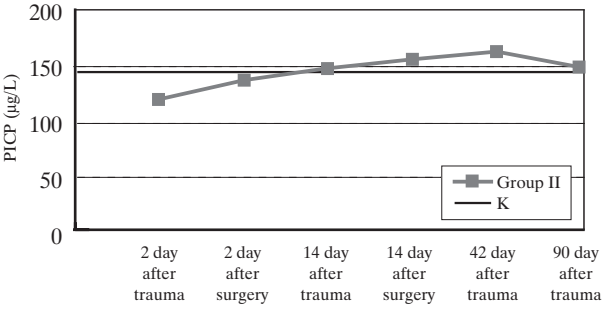

Table 4. **PICP concentrations in the blood serum in particular days of healing in I group of patients and control group (NS – statistically non-significant)**

The days of examination after trauma	2	14	42	90	Control group
PICP concentration (μg/L) ±SD	124.9 (±34.0)	151.2 (±36.4)	215.5 (±49.6)	126.9 (±34.2)	146.5 (±38.8)
Level of significance (p)	p<0.04	NS	p<0.0001	NS	-

Table 5. **Statistical differences between PICP concentrations in particular days of healing after trauma in I group of patients (NS – statistically non-significant)**

The days of examination after trauma	2:14	2:42	2:90	14:42	14:90	42:90
Level of significance (p)	p<0.005	p<0.0001	NS	p<0.0001	NS	p<0.0001

Table 6. **PICP concentrations in the blood serum in particular days of healing in II group of patients and control group (NS – statistically non-significant)**

The days of examination	2nd day after trauma	2nd day after surgery	14th day after trauma	14th day after surgery	42nd day after trauma	90th day after trauma	Control group
PICP concentration (μg/L) ±SD	121.2 (±32.2)	138.7 (±37.7)	149.3 (±34.9)	157.9 (±39.4)	165.9 (±36.0)	150.8 (±34.5)	146.5 (±38.8)
Level of significance (p)	p<0.01	NS	NS	NS	NS	NS	-

PICP

Group I. Blood serum of the patients showed PICP concentrations decrease on the 2nd day after the trauma, as compared to group K. Next two periods – on the 14th and 42nd day after the injury showed gradual increase in propeptide concentrations. The highest propeptide concentrations were noted on the 42nd day of fracture healing. In the last period, PICP concentrations again showed the decrease (*Fig. 3*).

The statistical analysis presented the decrease in PICP concentrations on the 2nd day and propeptide concentration increase on the 42nd day after the trauma, which were significant in relation to their concentrations in group K. However, differences of PICP concentrations observed between the controls and examined groups on other examination days were not statistically significant (*Tab. 4*).

Comparing PICP concentrations between test days we observed statistically significant increase in propeptide concentrations between 2:14, 2:42, and 14:42 days and statistically significant decrease between 42:90 days after the injury (*Tab. 5*).

Group II. Blood serum of the patients treated with stable osteosynthesis method showed lower PICP concentrations on the 2nd day after the trauma and on the 2nd after operation as compared to group K. The lowest values of propeptide concentrations were noted on the 2nd day after the injury. Since that time up to the 42nd day after injury, we observed constant increase in PICP concentrations. On the 90th day of examination, PICP decreased again and was at the level of PICP concentrations in group K. The highest PICP values were observed, as in non-operative patients, on the 42nd day of fracture healing (*Fig. 4*).

In comparison with PICP concentrations in group K, the decrease in propeptide concentrations on the second after trauma was statistically significant. Differences of PICP concentrations on other fracture healing days in patients operatively treated were not significant (*Tab. 6*).

Statistically significant elevation of propeptide concentrations between 2:14, 2:42, and 2:90 days was observed in group II, as compared with PICP concentrations on particular days after trauma (*Tab. 7*).

Figure 5. **ICTP concentrations in blood serum in I group of patients in examined days of healing and control group (K)**

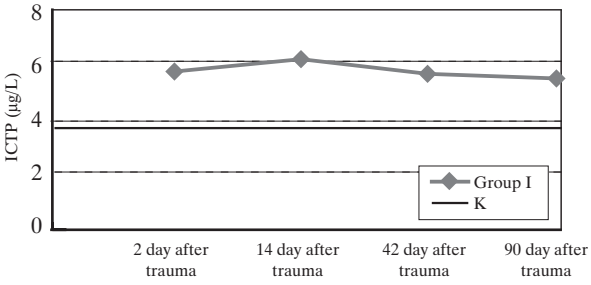

Figure 6. **ICTP concentrations in blood serum in II group of patients in examined days of healing and control group (K)**

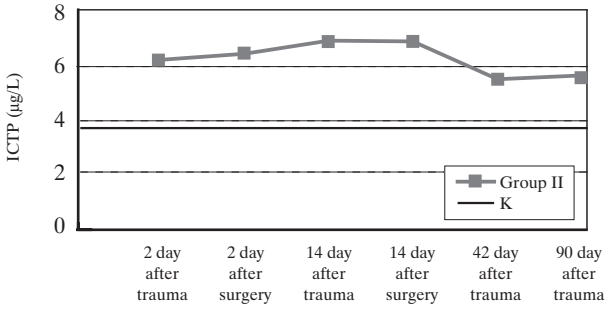

Table 7. **Statistical differences between PICP concentrations in particular days of healing after trauma in II group of patients (NS – statistically non-significant)**

The days of examination after trauma	2:14	2:42	2:90	14:42	14:90	42:90
Level of significance (p)	p<0.002	p<0.0001	p<0.001	NS	NS	NS

Table 8. **ICTP concentrations in the blood serum in particular days of healing in I group of patients and control group**

The days of examination after trauma	2	14	42	90	Control group
ICTP concentration (µg/L) ±SD	5.71 (±1.8)	6.12 (±1.56)	5.62 (±1.65)	5.42 (±1.54)	3.63 (±0.86)
Level of significance (p)	p<0.0001	p<0.0001	p<0.0001	p<0.0001	-

Table 9. **ICTP concentrations in the blood serum in particular days of healing in II group of patients and control group**

The days of examination	2nd day after trauma	2nd day after surgery	14th day after trauma	14th day after surgery	42nd day after trauma	90th day after trauma	Control group
ICTP concentration (µg/L) ±SD	6.12 (±1.8)	6.39 (±1.85)	6.82 (±1.74)	6.83 (±1.88)	5.39 (±1.52)	5.58 (±1.59)	3.63 (±0.86)
Level of significance (p)	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	-

Table 10. **Statistical differences between ICTP concentrations in particular days of healing after trauma in II group of patients (NS – statistically non-significant)**

The days of examination after trauma	2:14	2:42	2:90	14:42	14:90	42:90
Level of significance (p)	NS	NS	NS	p<0.001	p<0.005	NS

Positive correlation was not presented while comparing mean PICP concentrations on consecutive examination days in patients treated non-operatively and operatively.

ICTP

Group I. On particular test days of mandible fracture healing, elevated ICTP concentrations were observed in blood serum of patients treated with non-operative method, as compared to group K. The highest ICTP concentrations were noted on the 14th day after the injury. Next days presented ICTP concentrations close to telopeptide concentrations of the first period of the study (*Fig. 5*).

ICTP concentration increase, shown on each test day after the injury was statistically significant in relation to its concentrations in group K (*Tab. 8*). However, comparing ICTP concentrations between days of examination, we did not observe any statistical significance.

Group II. Elevated ICTP concentrations, compared to group K, were noted in blood serum of the patients with surgically treated mandible fracture, as it was in group I. Telopeptide concentrations increased gradually from the first day up to the 14th day after operation, reaching the highest concentration at this period. Next two periods of examination showed lowering of telopeptide concentrations and was at approximate level, however it was higher than telopeptide concentration in group K (*Fig. 6*). In relation to ICTP concentrations in group K, telopeptide concentration increase observed in mandible fracture healing was statistically significant (*Tab. 9*).

We compared ICTP concentrations between days after the trauma and revealed statistically significant lowering of ICTP between 14:42 and 14:90 days (*Tab. 10*). Positive correlation r=0.96 was observed, comparing mean ICTP of particular days of examination after the injury in patients of groups I and II.

However, we did not find any significant correlation between mean concentrations of examined markers of collagen type I and III metabolism in the course of mandible fracture healing in patients of both groups.

Discussion

An important component of fracture healing process is the synthesis of collagen types I and III in variable quantitative and temporary relations. Measuring products of collagen synthesis and degradation released into the blood, we can conclude about collagen metabolism type I and III in people.

In our studies, we evaluated concentrations of PIIINP, PICP, and ICTP in blood serum of men in the course of regular healing of mandible fractures, treated with non-operative (group I) and operative (group II) methods.

Significant elevation of PIIINP concentrations was observed in blood serum of the patients of both groups in all periods of fracture healing. In group I, the PIIINP concentrations was similar on the 2nd and 14th days after the trauma, on the 42nd day its concentrations significantly increased and was only slightly lower on the last day of examination.

The patients of group II revealed PIIINP concentrations increase on similar levels. Higher elevation of PIIINP concentrations in this group was observed only on the 2nd and 14th days after the operation.

The results point to the fact, that the trauma and/or the course of mandible fracture healing in men affect collagen type III metabolism increase and its activity is connected with the method of treatment.

The evaluation of bone fracture healing process based on PIIINP concentration measurement in blood serum has been the subject of only few studies [14-17]. Jerring et al. [14] and Kurdy et al. [16] obtained similar results to ours concerning group I, as far as the values and duration of maintenance of PIIINP concentration increase in blood serum in long bone healing with non-operative method were concerned.

Two basic mechanisms of reconstruction of bone tissue continuity can be distinguished in fracture healing: so called spontaneous concrescence and primary concrescence [22,23]. Fracture healing through spontaneous concrescence occurs in conditions of limited mobility of fragments, which can be obtained while applying non-operative methods to fracture treatment. Spontaneous concrescence is produced due to participation of osteogenic cells of internal layer of periosteum, osteogenic cells of endosteum, and bone marrow [22,23]. The exact adhesion and "ideal" immobilization of fragments, which can be obtained by applying stable osteosynthesis with compression are the conditions of reconstructing the continuity of fractured bone through primary concrescence. Giving up compression, exact adhesion of the plate to the bone and its elasticity in fracture healing was the basis of introducing indirect concrescence. This way of healing, the reconstruction of bone continuity occurs through primary accretion (under the plate) and that similar to spontaneous concrescence (on the side opposite to the plate) [23].

Our studies show no correlation between mean PIIINP concentrations obtained on particular days of examination after injury in patients treated with non-operative and operative methods. That can suggest that fracture healing treated with these methods occur through different mechanisms.

In opposition to present studies, Jerring et al. [15] observed significant increase in PIIINP concentrations in regular fracture healing of tibial bone treated non-operatively and operatively on the 1st and 2nd weeks after injury. Further examinations presented propeptide concentrations remaining elevated up to the end of the study i.e. on the 26th week after the trauma. The authors did not state any differences in dynamics of PIIINP concentration changes depending on fracture healing treatment method. These discrepancies might be due to the fact, that results of PIIINP concentrations referred to the 1st day after the injury and not to the control group, the type of bone fracture, small number of examined patients, different age and sex [15].

The results cited authors seem to confirm our conclusion that the course of regular mandible fracture healing may affect collagen type III metabolism increase.

A mature bone tissue contains a small amount of collagen type III, mainly in blood vessels and Havers canals [3]. In the course of long bone fracture healing in animals, type III collagen appears earlier and is more abundant than collagen type I [3,9]. The authors' results may thus account for dynamics of PIIINP concentration changes observed in our studies.

The role of type III collagen in fracture healing has not been determined yet. It is assumed that type III collagen may play a role of "organic framework" which enables migration, bone cell adhesion and blood vessels in-growing [3,9]. Beside fracture regeneration process, type III collagen is synthesized as the first in skin wound healing [24-27] and tendon healing [3]. It is assumed that type III collagen is a significant component of most body regenerative processes [3,10,24-28].

PIIINP concentrations determined in blood serum reflect both synthesis and degradation of type III collagen. It is impossible to determine proportions of PIIINP that comes from synthesis or degradation of type III collagen on the basis of propeptide measurement in blood serum and using available RIA kits. To solve the problem we should know molecular structure and metabolism of PIIINP propeptide and work out specific methods enabling separate evaluation of collagen type III synthesis and degradation.

Significant PIIINP concentration increase in both groups of patients observed on the 2nd day after the trauma can be the result of general reaction to the injury from both the skeletal system and soft tissues. Waydhas et al. results seem to confirm the assumption. They observed significant elevation of PIIINP in blood serum of patients with severe bodily injuries in the period from 3 to 14 days after trauma. Moreover, they pointed to the fact that PIIINP concentration was decidedly higher in patients with coexisting bone fracture [28].

Comparing our studies to fracture healing phases through spontaneous concrescence mechanism we can only suppose that PIIINP concentration increase on the 2nd day after the injury can occur due to the propeptide release from damaged blood vessels and soft tissues at the side and surroundings of the fracture. Considering the order of appearance of type III and I collagen in animal models, we can assume that PIIINP concentrations

observed on the 14th day of our studies is a result of collagen type III synthesis. As collagen type I is the only component of mineralization process in bone organic matrix [3,29], observed increase in PIIINP concentrations on the 42nd day, i.e. in the period of clinical mandible concrescence, can be the result of degradation of earlier synthesized type III collagen. Wen et al. studies stated the necessity of the replacement of collagen type III by type I in this period of bone fracture healing. Their studies showed, that the lack of exchange in quantitative relations of both types of collagen is the cause of bone concrescence disturbances. The disturbances are reflected by insufficient mineralization in microstructural evaluation [29].

Multimaki et al. studies presented the highest mRNA level for type I collagen in the period of callus remodelling and lamellar bone formation. The level of mRNA for collagen type III was minimum in this period of healing [2]. Having the data in mind, we can only assume, that high PIIINP concentration observed on the 90th day after mandible fracture is the result of type III collagen degradation. It also points out, that the process of fracture healing is in the course of duration. As far as mandible is concerned, the period of callus reconstruction is 1 year [30].

Assuming the possibility of mandible fracture healing in patients of group II through the primary and/or indirect concrescence, the explanation of PIIINP increase on the 14th, 42nd, and 90th days after the trauma requires further experimental studies to determine the kind and possible quantitative relations of collagen types I and III.

Analyzing the results of PICP concentrations in serum of patients of groups I and II in the course of mandible fracture healing, its significant lowering was observed on the 2nd day after injury. Since that time, up to the 42nd day after the trauma, constant increase in serum PICP concentrations of patients of both groups was observed. The highest PICP concentrations were also presented in both groups of patients on the 42nd day. It should be noticed, that absolute values of PICP concentrations were evidently higher in group I than in group II. Statistically significant PICP concentration increase was shown, as compared to the controls, only in group I. The 90th day did not give any particular differences of PICP concentrations in both groups as far as propeptide concentrations in the control group were concerned.

The results point to the fact that regular course of mandible fracture healing in men can affect changes of PICP concentrations and the dynamics of these changes, like in the case of PIIINP concentrations, is connected to the method of treatment.

Kurdy et al. [16] observed the dynamics of PICP concentration changes similar to ours in group I. Their study concerned patients with regular healing of tibial fracture treated non-operatively. However, Jerring et al. did not notice PICP concentration lowering in the first days of regular healing of radial bone fracture [14] and tibia [15] in patients treated non-operatively. They observed significant increase in PICP concentrations on the 2nd and 5th weeks of radial bone healing [14] and on the 2nd and 6th weeks of tibial bone healing [15]. PICP concentrations obtained in the course of long bone fracture healing referred to the 1st day of examination (i.e. the day of admission) Jerring et al. [14,15], and not to propeptide concentrations in the control group. Analogically, the relation of group I results and PICP concentrations on the 2nd day after the trauma (the first examination) and differences of duration of mandible and particular long bone fracture healing show our results comparable to those of Jerring et al. [14,15].

Jerring et al. observed significant PICP concentration increase in single cases of delayed concrescence of long bones on the 1st and 2nd weeks after the injury [15]. Other studies of delayed concrescence of long bones [17] showed significant PICP concentration increase on the 5th week. However, they observed statistically significant decrease in propeptide concentrations on the 10th and 20th weeks [15,17]. The cited authors conclude of usefulness of PICP concentration determination as a non-invasive method to evaluate regular and disturbed processes of fracture healing in people.

There was no positive correlation in present studies between mean concentrations of PICP and PIIINP if patients treated non-operatively and operatively. It can suggest that fracture healing treated with non-operative method occurs through spontaneous concrescence and with stable osteosynthesis through primary and/or indirect accretion [18,23]. The mechanism of healing through primary concrescence, bone continuity reconstruction is based on "exaggerated reconstruction" [23]. Conditions, which have to be fulfilled in this method of healing exclude or limit the participation of hematoma and soft tissues in the closest surrounding in bone regeneration. They also eliminate beneficial activity of mechanical stress on bone tissue [19,20,23]. It can be reflected in our studies in differences of PIIINP and PICP concentration changes observed in both groups. Group I revealed dynamics of these changes more evidently than group II. Suggestions of our studies are in accordance with Lotz et al. observations [18] concerning PICP concentrations in serum of patients with femoral bone fracture treated with stable osteosynthesis method with compression as well as other surgical methods that enable healing through spontaneous concrescence.

Jerring et al. [15], unlike Lotz et al. [18] and our data, did not observe differences in dynamics of PICP concentrations in regular tibial fracture healing, treated operatively and non-operatively, depending on treatment method. The differences can occur due to relation of PICP concentrations to the 1st day after the trauma and various operative methods used in the treatment.

Our studies revealed significant decrease in PICP concentrations on the 2nd day after injury in patients of both groups. Taking into account that PICP concentrations in blood serum of young men are maintained mainly due to continuous metabolism of type I collagen in the skeletal system [8], PICP concentration lowering in our studies was not expected in this period. Thus, a question arises whether PICP concentration decrease in blood serum can be the result of general reaction to the trauma and/or to fracture. Protein biosynthesis decrease is one of constant component of general reaction to injury. The period changes of existence in blood serum connected with the reaction of the acute phase in people is described in the range of 1 to 4 days after the injury [30]. PICP concentration lowering observed in our studies fits the time intervals. Kurdy et al. [16]

tried to explain the decrease in PICP concentrations in blood serum of patients with "temporary suppression" of osteoblast activity in response to the trauma, which is the bone fracture.

According to observed PIIINP and PICP concentrations in blood serum of men in the course of mandible fracture healing, PICP concentration increase appears later than PIIINP. Antonowicz showed similar dynamics of PICP concentrations in rat serum in experimental mandible fracture healing [1]. It is in accordance with other authors' results that evaluated collagen types in models of bone fractures in animals using polyclonal antibodies [3,9], immunofluorescent and histological examinations [3] and the expression of collagen genes [2]. It indicates the similarity of type I and III collagen appearance in regeneration of people and animals bone fractures. The coexistence of various types of collagen can suggest that they complement each other as for functioning in the course of fracture healing [9,19-21]. However, their role during healing process has not been described in details yet.

It should be noticed that PIIINP and PICP are not tissue specific markers. The propeptide release takes place during extracellular changes of collagen of the skin, ligaments, and fascia [5,8,10]. Thus, we should consider other than bone tissue sources of PIIINP and PICP. It is stressed that in generally healthy people, bone metabolism is faster than that of other kinds of connective tissue. Thus, it is assumed that bone tissue metabolisms characterized by PICP concentrations in these cases [8] and that the participation of PICP and PIIINP, other than of bone origin, can be increased only in diseases with intensive fibrosis [8,31,32].

Significant increase in PICP and PIIINP concentrations in blood serum was observed only after extensive surgeries, mainly of the abdominal cavity [24,26]. In the course of soft tissue wound healing, e.g. after hernia operation, there were no significant changes of the propeptide concentrations [26]. In our studies in both groups, mandible fractures were open fractures and had contact with the oral cavity environment through mucous membrane injuries or only through periodontal fissure. In compliance with the rules, sections of soft tissues in the region of the facial skeleton were very economical. Moreover, we used two additional determinations of markers concentrations on the 2nd and 14th day after surgery to evaluate the influence of soft tissues on PICP and PIIINP blood serum concentrations of patients treated operatively. The values obtained on these days and studies mentioned above show that the influence of soft tissue healing on PICP and PIIINP concentrations in blood serum in our studies were not significant.

The significant increase in ICTP concentrations was observed in both groups in all periods of mandible fracture healing as compared to the controls. We compared the dynamics of ICTP concentrations in both groups and revealed that telopeptide concentration increase from the 2nd to 14th days after the trauma in group II was evidently higher than in group I. In consecutive two periods after the injury (the 42nd and 90th days), serum ICTP concentrations were similar in both groups.

The results can suggest that the trauma and/or the course of healing in men influence serum ICTP concentration increase regardless the method of treatment.

A similar dynamics of ICTP concentration elevation, observed in our studies, was also presented by Jerring et al. [15], Kurdy et al. [16], and Lotz et al. [18] in the course of long bones healing. Their results point to the fact that fracture and regular course of healing of a relatively small bone, the mandible, causes similar changes of ICTP and other markers of collagen metabolism concentrations as in the case of long bones.

In delayed concrescence of long bones, ICTP concentrations did not differ significantly from telopeptide concentrations observed in regular healing process of these bones [15,17].

In contrast to PIIINP and PICP, concentrations of ICTP were on similar levels in both examined groups. It was confirmed by the positive correlation.

The influence of trauma and/or fracture healing on the skeletal system or a fractured bone has been the subject of few studies on animals and people. Einhorn et al., on the basis of histomorphometric examination on animals, stated general reaction of the skeletal system, caused by long bone fracture, to the trauma. The skeletal system reaction, i.e. the elevation of bone metabolism index, was almost immediate and lasted up to the 3rd week [33]. Osteopenia was diagnosed in a fractured extremity histologically and radiologically in animals [19,20,34]. People also revealed osteopenia, diagnosed histomorphometrically [35] and densitometrically in a fractured extremity. Bone mineral density decrease maintained for several months after the fracture [36]. The results can confirm ICTP concentration increase observed in our studies.

ICTP and PICP concentrations analysis showed that two first weeks of mandible fracture healing are characterized by degradation increase and type I collagen synthesis decrease. The concentrations of PICP and ICTP, observed in group I on the 42nd day after injury, can suggest the superiority of type I collagen synthesis over its degradation. On the 90th day in group I and on the 42nd and 90th days in group II, both processes are in balance.

Resorption and osteogenic processes are closely linked in regular conditions [8]. However, in metabolic bone diseases, there is no such linking. The superiority of resorption over formation leads to eventual loss of bone mass [6,8].

The results of cited authors [15,16,18] and our studies show lack of linking of synthesis and degradation of collagen type I in early periods of regular bone healing. Kurdy et al. [16] observation can be partially explained by so called regional acceleratory phenomenon (RAP) [19,20].

Based on the data it can be assumed that early loss of type I collagen in patients with bone fracture is not a specific feature connected with the process of mandible fracture healing but general reaction to the trauma. Other authors have the same opinion [15,17], however, the subject requires further investigation.

Moreover, we should take into account that age, sex, condiments, certain drugs, coexisting diseases (mainly metabolic) [8,37] as well as paradontal diseases [38] have a great impact on the concentration of markers of collagen type I and III metabolism determined in blood serum. The concentrations of resorption and bone formation markers in men are highest between 20 and 30 years of age and correspond to the peak of bone mass [37]. The above-mentioned data were the basis for conducting the study on men in one age interval and

determining other strict qualifying criteria described in Material and methods.

Conclusions

1. Regular process of mandible fracture healing in men in various periods occurs with PICP, PIIINP, and ICTP concentration changes in blood serum. It indicates that the trauma and/or normal bone healing finds its reflection in changes of examined collagen metabolism markers type I and III in men's blood serum.

2. As the evaluation of marker concentration changes show that, mandible fracture healing treated non-operatively is a more dynamic process than stable osteosynthesis method applied.

3. Lack of positive correlation PIIINP and PICP concentration in blood serum of patients with mandible fracture treated non-operatively and operatively can suggest, that fracture healing treated with conservative-orthopedic methods occurs through spontaneous concrescence while treated with stable osteosynthesis through primary and/or indirect concrescence. However, the subject requires further investigation.

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Serum levels of interleukin-18 (IL-18) and soluble interleukin-2 Receptor (sIL-2R) in lung cancer

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Abstract

Purpose: We evaluated the clinical usefulness of interleukin-18 (IL-18) and soluble interleukin-2 receptor (sIL-2R) during chemotherapy of lung cancer in relation to the histological type of the tumor, clinical stage, response to therapy and time survival.

Material and methods: Serum levels of IL-18 and sIL-2R were determined in 73 patients (62 males; mean age 64 years; 41 with non-small cell lung cancer-NSCLC, 32 with small cell lung cancer-SCLC); 12 healthy subjects served as controls. To determine IL-18 serum concentrations (Elisa), venous blood samples were collected from each patient before and after chemotherapy.

Results: The mean serum IL-18 level in all patients with lung cancer was significantly higher compared with healthy volunteers ($p=0.0001$; NSCLC vs control $p=0.0001$; SCLC vs control $p=0.004$). In NSCLC group with stage IV the mean IL-18 level was significantly higher than those with stage IIIB ($p=0.04$). Regardly to tumor stage as well as in relation to response to therapy, no significant differences in IL-18 were observed. Using cut-off serum IL-18 concentration of 319.6 pg/ml, the prognoses of the two groups were different, but it was not statistically significant. The serum levels of sIL-2R in NSCLC patients were significantly higher than in controls ($p=0.018$). There were no significant differences in serum sIL-2R levels in relation to clinical stage of lung cancer and response to therapy. The cut-off value between high and low serum sIL-2R concentration was defined as 582.27 U/ml. The difference in survival rate between the high and low sIL-2R groups was not significant.

Conclusions: Serum IL-18 and sIL-2R levels can be useful in clinical practice, but their practical significance needs further studies.

Keywords: interleukin-18 (IL-18), soluble interleukin-2 receptor (sIL-2R), small cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), chemotherapy.

Introduction

Interleukin 18 (IL-18) is a novel immunoregulatory cytokine that was known previously as interferon-gamma-inducing factor (IFN-γ) [1]. This cytokine is produced by activated macrophages, keratinocytes, Kupffer cells, intestinal epithelial cells, and osteoblasts [1]. The high levels of IL-18 have been detected in cancer patients (hematologic malignancies, gastric carcinoma, breast carcinoma) [2,3,4]. There are not data about IL-18 in lung cancer. IL-18 enhance the development of T-helper cells (Th1) that seems to play a crucial role in the generation of antitumor immunity [5]. IL-18 induces the production of IL-2 from Th1 cells [6]. The combined use (in vitro) of these two cytokines synergistically enhance the proliferation, cytolytic activity, and interferon-gamma production of peripheral blood mononuclear cells [7]. The activated mononuclear cells can release a soluble form of interleukin 2 receptors (sIL-2R) in the blood. Serum sIL-2R level is a sensitive and quantitative marker of circulating peripheral blood mononuclear cell activation [8]. This molecule acts as an antagonist of IL-2-mediates responses [9]. sIL-2R levels reflect T-cell activation and correlate with the disease activity [10,11]. To determine the clinical importance of IL-18 in lung cancer patients in the current study, we measured serum IL-18 and sIL-2R levels. We were curious about the correlation

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between IL-18 and sIL-2R serum levels. We analyzed the levels of IL-18 and sIL-2R in lung cancer in relation to the histological type of the tumor, clinical stage, response to therapy and time survival for patients.

Materials and methods

Patients

The study included 73 patients with carcinoma of the lung, who where admitted to the Department of Pneumonology, Medical Academy in Bialystok from 1999 to 2002. They consisted of 62 males and 11 females (mean age of 64.0 years; ranged 29-78). The tumors were histologically classified as adenocarcinoma in 8 cases, squamous cell carcinoma in 33, and small cell carcinoma in 32. None of the patients suffered from infectious, allergic, autoimmune, or other systemic diseases such as diabetes mellitus and hypertension. The patients had not been previously treated with chemotherapy. The control group for serum IL-18 concentrations comprised 12 healthy volunteers (10 males) with mean age of 61 years. There were no significant differences in age and sex between patients and controls. All patients had a history of smoking.

Methods

Before receiving treatment, patients underwent standard staging procedures consisting of physical examination, serum chemistry examination, bronchoscopy, chest CT scan and ultrasonography of abdomen. Further imaging techniques were used when required clinically. The clinical stage of non small cell lung cancer (NSCLC) was assigned according to the International Union Against Cancer (TNM classification). The classifications of small cell lung cancer (SCLC) were made according to the Veterans Administration Lung Cancer Study Group (LD-limited disease; ED-extensive disease). After staging, the patients were placed on cisplatin or platin-derived chemotherapy, which was coupled with radiotherapy in the locally advanced forms. Standard criteria for objective response to therapy were used (WHO guidelines). To exclude the possible interference of chemotherapy, subsequent blood samples were obtained at least 28 days after the last administration of cytotoxic drugs. To determine IL-18 serum concentrations, venous blood samples were collected from each patient before and after IV cycles chemotherapy (some of the patients underwent later radiotherapy). Serum samples were obtained by centrifugation and stored at -80°C until assayed. Serum IL-18 concentrations were measured by a single labolatory with an enzyme immunoassay (Human IL-18 ELISA Kit; MBL, Japan; sensivity: < 12.5 pg/ml) according to the manufacturer's instructions. The sera were assayed for sIL-2R with an enzyme – linked immunosorbent assay using Cellfree Human Elisa sIL-2R Kits (Endogen, USA; sensivity: < 24 U/ml). All samples were assayed in duplicate.

Statistical analysis

Data were presented as mean ± 1 SD or median (range), depending on their normal or skewed distribution provided by Shapiro-Wilk's W test. Data for IL-18 and sIL-2R concentrations in serum samples from healthy subjects and from patients with lung cancer were analyzed using Student's T-test for independent samples. Differences among groups of patients before and after chemotherapy were determined using Student's T-test for dependent samples. In the case of skeweew distribution the data were analysed using Wilcoxon's-test and Mann-Whitney's U-test for unpaired data. Correlation between the parametres were calculated by the Spearman's and Pearson's rank tests.

Survival curves were generated using the Kaplan-Meier's method, and the significance of the difference in survival rates was determined by the log-rank test. Multivariate analysis was performed using a Cox's proportional hazards model. All patients with lung cancer were divided into two groups according to their IL-18 and sIL-2R serum levels. The cut-off point was set at 319.6 pg/ml for IL-18 and 582.27 U/ml for sIL-2R. The cut-off points represented the mean ± 1 SD of serum IL-18 and sIL-2R values in healthy volunteers.

All p values were two-tailed, and values less than 0.05 were considered statistically significant. Computations were performed using Statistica 6.0 for Windows (StatSoft Inc., Tulsa, Okla, USA).

Results

Serum IL-18 and sIL-2R levels in healthy volunteers and patients with lung cancer

As shown in *Tab. 1*, the serum IL-18 levels in 73 patients with lung cancer were significantly higher compared with the 12 healthy volunteers ($p = 0.0001$; NSCLC vs control $p = 0.0001$; SCLC vs control $p = 0.004$). There were no significant differences in serum IL-18 levels with regard to patient age or gender, or histologic type.

The serum sIL-2R levels in lung cancer patients were significantly higher than in control ($p = 0.023$; NSCLC vs control $p = 0.018$) (*Tab. 1*). The difference between patients with NSCLC and SCLC was not significant and the levels of sIL-2R in SCLC did no differ significantly from control group.

There was a positive correlation between IL-18 and sIL-2R in NSCLC group ($p = 0.020$; $r = 0.360$).

Serum IL-18 and sIL-2R levels in relation to clinical stage of the tumor

In NSCLC group (*Tab. 1*) the mean IL-18 level of patients with stage IV was significantly higher than those with stage IIIB ($p = 0.04$). No significant differences in serum IL-18 levels with regard to tumor stage of SCLC were observed (*Tab. 1*).

There were no significant differences in serum sIL-2R levels in relation to clinical stage of NSCLC and SCLC (*Tab. 1*).

Serum IL-18 and sIL-2R levels in relation to response to therapy

There were no significant differences in serum IL-18 levels in relation to response to therapy (*Tab. 2*). No significant differences in serum sIL-2R levels with regard to response to therapy were observed (*Tab. 2*).

Table 1. **Serum IL-18 and sIL-2R levels in lung cancer patients and controls**

Disease stage		before chemotherapy (p-VALUE VS CONTROLS)	after chemotherapy (p-VALUE VS CONTROLS)	Controls (n = 12)
Lung carcinoma patients (n = 73)	IL-18 sIL-2R	390.9 ± 151 p = 0.0001 602.3 (65 - 2016) p = 0.023	498.2 ± 314 p = 0.00006 840.8 (41 - 7233) p = 0.014	227.3 ± 92 516.7 (281 - 596)
NSCLC (n = 41)	IL-18 sIL-2R	411.0 ± 144 p = 0.0001 625.6 (65 - 2016) p = 0.018	494.5 ± 316 p = 0.00004 614.7 (41 - 4431) p = 0.012	
III A (n = 4)	IL-18 sIL-2R	341.3 ± 80 613.9 (471 - 640)	524.7 ± 150 992.9 (640 - 1419)	
III B (n = 12)	IL-18 sIL-2R	338.8 ± 118 # 574.7 (65 - 5016)	384.9 ± 114 509.4 (41 - 1568) *	
IV (n = 25)	IL-18 sIL-2R	442.8 ± 144 ## 696.1 (153 - 1932)	475.1 ± 209 614.7 (330 - 2060)**	
SCLC (n = 32)	IL-18 sIL-2R	370.9 ± 157 p = 0.004 567.5 (137 - 1889)	506.1 ± 309 p = 0.005 589.3 (169 - 7233)	
LD (n = 13)	IL-18 sIL-2R	435.4 ± 189 567.5 (313 - 978)	526.8 ± 249 560.9 (169 - 959)	
ED (n = 19)	IL-18 sIL-2R	326.9 ± 116 562.3 (137 - 1889)	489.1 ± 209 603.4 (265 - 7233)	

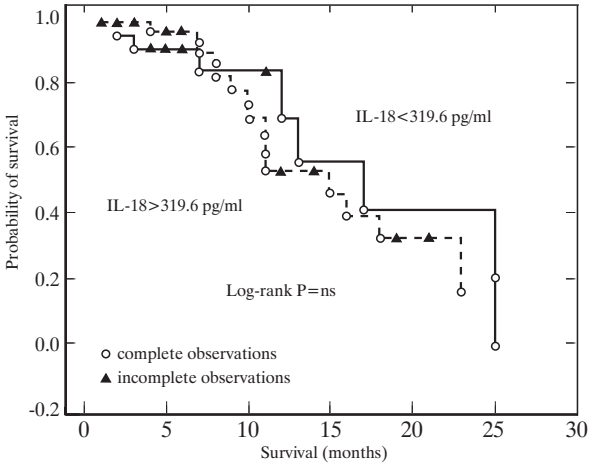
Abbreviations: IL-18 – interleukin 18 (pg/ml); sIL-2R – soluble interleukin 2 Receptor (pg/ml); # vs ## p = 0.04 ; * vs ** p = 0.04

Table 2. **Values of IL-18 and sIL-2R before and after chemotherapy of lung cancer patients**

NSCLC (n = 41)	**PR (n = 14)**		**NC (n = 16)**		**PD (n = 11)**	
	before chemotherapy	after chemotherapy	before chemotherapy	after chemotherapy	before chemotherapy	after chemotherapy
IL-18 sIL-2R	386.2 (230 – 537) 613.9 (257 - 946)	447.4 (184 - 977) 550.4 (137 - 1419)	407.9 (171 – 607) 523.9 (65 - 1315)	390.6 (269 - 1091) 603.8 (41 - 2060)	349.3 (135 – 652) 858.6 (129 - 2016)	380.9 (154 -718) 698.2 (241 - 1568)
SCLC (n = 32)	**PR (n = 17)**		**NC (n = 4)**		**PD (n = 11)**	
	before chemotherapy	after chemotherapy	before chemotherapy	after chemotherapy	before chemotherapy	after chemotherapy
IL-18 sIL-2R	334.8 (146 - 942) 525.6 (137 - 1889)	414.1 (163 – 1265) 582.0 (265 - 7233)	363.5 (234 - 521) 978.8 (654 - 1195)	616.9 (287 - 1109) 690.9 (554 - 1050)	381.1 (195 - 652) 567.5 (426 - 727)	453.5 (164 - 1336) 509.4 (169 - 810)

PR – partial response; NC – no change; PD – progressive disease

Figure 1. **Probability of survival for lung cancer patients in relation to their serum IL-18 levels**



Serum IL-18 and sIL-2R levels in relation to time survival for patients

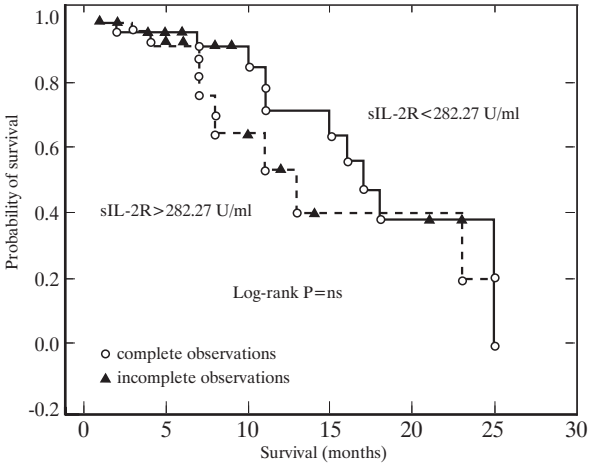
When all patients with lung cancer were divided into high and low groups using cut-off serum IL-18 concentration of 319.6 pg/ml, the prognoses of the two groups were different, but it was not statistically significant (*Fig. 1*).

The cut-off value between the high and low serum sIL-2R concentration was defined as 582.27 U/ml. There was no statistical difference in survival rate between the high and low sIL-2R groups (*Fig. 2*).

Discussion

IL-18 enhanced the immune defense against tumor cells by activating and inducing the production of IFN-γ [12]. In addition to its IFN-γ- enhancing capacity, IL-18 also augments the cytotoxic activity of natural killer (NK) and T-cells and enhances

***Figure 2*. Probability of survival for lung cancer patients in relation to their serum sIL-2R levels**



their production of other proinflammatory mediators such as TNF-α, IL-1β, IL-8 [13,14]. IL-18 elicits antitumor immunity in the murine system by inhibiting tumor angiogenesis, reducing tumorigenesis, and inducing apoptosis in tumor cells [12,13,15, 16]. Although elevated serum IL-18 levels have been reported in patients with renal cancer [17], esophageal cancer [18], breast cancer [4], hematologic malignancies [19], the clinical impact of IL-18 remains unclear in patients with solid tumors. To our knowledge, the current study is the second to report serum IL-18 levels in patients with lung cancer. The first was Lissoni at al., who observed no significant differences in IL-18 mean levels between non-metastatic patients and controls [20]. The patients with metastases of lung cancer had higher IL-18 levels than controls [20]. The same observations were made by Lissoni in patients with gastrointestinal tumors [20].

Our study showed that all patients with lung cancer had higher IL-18 serum levels than healthy volunteers. There were NSCLC patients with stage IIIA, IIIB, and IV in our study. The mean serum IL-18 level of patients with stage IV (i.e. the metastatic patients) was significantly higher compared with patients who had stage IIIB.

Our results are in agreement with increased IL-18 levels evidenced in patients with advanced neoplasma disease as shown by Gunel and Merendino separately [4,21]. They showed that serum IL-18 levels were higher in the metastatic patients compared with the nonmetastatic ones. They measured serum IL-18 levels in breast carcinoma patients. In Merendino opinion IL-18 could act as a marker for metastatic breast cancer [21].

We did not observed significant differences in serum IL-18 levels with regard to tumor size and lymph node metastases. The same observations were made by Kawabata and coworkes in patients with gastric carcinoma [3].

In our study there were no statistical differences in SCLC group between stage ED and LD. These observations confirm that different mechanisms are responsible for IL-18 increase in solid tumors. IL-18 is produced mainly by macrophages, but it is not clear which cells synthesize IL-18 in different circumstances and which conditions favor IL-18 production. IL-18 production may be induced in response to tumor cells or other factors related to tumor growth [3,4]. The high serum IL-18 levels in lung cancer patients may reflect the degree of defense mechanisms against tumor growth and metastasis.

In our studies, there were no significant differences in respect to response to therapy. To our knowledge, the current study is the first to report serum IL-18 in relation to response to therapy of the lung cancer. Kawabata et al. in gastric carcinoma patients showed that serum IL-18 level after surgical resection was significantly lower than before surgery [3]. The mean serum IL-18 level after surgery was similar to that of the controls [3]. These observations suggested that measuring of IL-18 can be useful in monitoring treatment of tumors. However, the clinical impact of IL-18 remains unclear in lung cancer patients who underwent chemotherapy.

It has been reported that elevated serum IL-18 levels also were observed in patients with gastric carcinoma, colorectal carcinoma, non-hodgkin's lymphoma and was associated with a poor prognosis [2,3,22]. Akahiro et al. [23] showed that IL-18 serum levels were correlated with overall survival, although they were shown not to be an independent prognostic factor. In our study, although it was without statistical significance, the patients with lung cancer in each stage who had high serum IL-18 levels also experienced poorer survival compared with patients who had low serum IL-18 levels.

IL-18 synergizes with IL-2 to enhance cytotoxity, IFN-γ production, and expansion of NK cells [7]. Activation of T lymphocytes leads to the expression of interleukin 2 receptor on the cell surface as well as the release of soluble IL-2R molecules into the circulation [11]. T lymphocytes are the predominant IL-2R bearing cells and hence serum sIL-2R level provides a satisfactory indicator of T-cell activation in vivo [10,11]. A soluble form of the IL-2 receptor, consisting of an incomplete variety of alpha chain, retains the ability to bind IL-2 [24]. The high levels of sIL-2R shown in many instances where IL-2-dependent functions are coincidentally impaired, suggest that the molecule may act as an antagonist of IL-2-mediated cell response. Abnormally high levels of sIL-2R have been described in both hemolymphopoietic tumors and solid tumors [25,26]. A lot of studies have revealed increased sIL-2R levels in lung cancer [9,11,27,28]. sIL-2R levels reflect T-cell activation and correlate with the disease activity [10,11].

Our studies showed that we observed a correlation between IL-18 and sIL-2R levels in NSCLC group. Our results are in agreement with observations made by Son et al. [7]. They showed that combined use of IL-18 and IL-2 substantially enhanced the cytolytic activity of peripheral blood mononuclear cells [7].

The behaviour of serum sIL-2R levels in lung cancer patients is controversy.

In our studies serum levels of sIL-2R were significantly higher in cancer patients than in controls. We did not observe significant differences in respect to clinical stage of NSCLC according to TNM classification. Our results are in agreement with studies showed by Orditura et al. [25].

It has been reported that the measuring of sIL-2R can be useful in monitoring chemotherapeutic treatment [9,25]. A reduction in sIL-2R serum levels is generally associated with

a full or partial response to chemotherapy, while they increase during a progression of the disease in spite of the treatment [10]. Conversely, we did not observe significant differences in serum sIL-2R levels with regard to response to chemotherapy.

In some clinical studies, serum concentration of sIL-2R may be a prognostic factor in patients with lung cancer [9,21]. Tisi et al. showed, that the evaluation of sIL-2R serum levels in the postoperative period may have prognostic importance in predicting the risk of early relapse in patients with operable NSCLC [29]. The evidence of a significant association between sIL-2R increases in the postoperative period and early recurrence rate suggested that host immune factors played at least some role in the prognosis of lung neoplastic disease [29]. It has been reported that the patients with abnormal levels of sIL-2R had a worse prognosis, and this could be useful in prognostication [30]. In our study, although it was without statistical significance, the patients with lung cancer in each stage who had high serum sIL-2R levels also experienced poorer survival compared with patients who had low serum sIL-2R levels.

According to our data there were no significant differences in serum sIL-2R levels with regard to histological type of the tumor. The same observations were made by Buccheri, Brunetti and Sarandakou separately [11,10,27]. They noticed a higher sIL-2R concentration in the serum of SCLC patients as compared to that of NSCLC patients, whereas Yano et al. came to opposite conclusions [28]. Moreover in the case series studies by Lissoni et al. there was no distinction in the group of SCLC patients between limited and extended disease [22]. In our study we have made the same observations like Lissoni et al. [26]. We showed that the levels of sIL-2R in SCLC did not differ significantly from healthy volunteers. In NSCLC patients the levels of sIL-2R were significantly higher than in controls.

These observations confirm that different mechanisms are responsible for sIL-2R increase in solid tumors. The leader hypothesis links the sIL-2R increase in solid tumors to the release from activated cells (lymphocytes, macrophages) to express IL-2R during immune-reactions against cancer spreading [25]. Contrary, the increase of sIL-2R levels could be seen as a consequence of a lymphocyte functional damage in patients with solid tumors. Alternatively, it could contribute to the condition of immuno-depression in solid tumors, by means of the competition with cell surface receptor for IL-2 [25].

Concluding, the measuring of IL-18 and sIL-2R can be useful in clinical practice. The clinical significance in monitoring chemotherapy, prognosis of lung cancer needs further studies.

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The spontaneous and stimulated nitroblue tetrazolium (NBT) tests in mononuclear cells of patients with tuberculosis

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Abstract

Purpose: The aim of this study was to evaluate the ability of the NBT reduction by non- and BCG-stimulated monocytes isolated from peripheral blood persons with pulmonary tuberculosis (TB) before treatment, after two-month antituberculosis therapy and in an inactive stage of this disease.

Material and methods: The spontaneous and induced NBT tests were done in 24 healthy individuals and 59 patients with pulmonary tuberculosis: 33 before antituberculosis treatment and 26 with inactive stage of TB. Mononuclear cells were isolated from peripheral blood by the Bøyum method and indentified by histochemical assay. The abilities of non- and BCG-stimulated monocytes of NBT reduction were estimated by the method according to Park with Szczylik modification.

Results: In an active state of TB and after 2 months treatment, the non- and BCG-stimulated monocytes capacity to reduce NBT was found to be significantly increased in comparison to controls. The NBT test parameters in the absence of cell stimulation and after administration of BCG were comparable in active TB and after two-month treatment. In an inactive TB, the ability of NBT reduction by non- and BCG-stimulated monocytes was comparable to the controls. The stimulation of mononuclear cells accompanied by the significantly higher capacity of monocytes to reduce NBT in controls and in TB patients with post-tuberculous changes in the lungs.

Conclusions: These results of the spontaneous and induced NBT tests adequately reflect the status of the host's specific reactivity during tuberculosis and can be simple, cheep and useful for a monitoring antituberculosis treatment.

Key words: NBT test, monocytes, tuberculosis.

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Introduction

Tuberculosis (TB) is still an important world health problem, and it is estimated that about one third of the earth's population has been infected with Mycobacterium tuberculosis [1,2]. Each year, there are about 2 million deaths due to TB [1].

It is still not clear why only approximately 1 in 10 of those infected will progress to active disease during their lifetime, however, only a minority of them possess a risk factor, such as advanced age, alcohol abuse, immunosuppressive therapy, diabetes or an acquired immuno-deficiency syndrom (AIDS) [3].

Tuberculosis is a disease known to be associated with various immunological alterations, including some changes in the cell- and antibody- mediated immunity [4]. The monocytes/macrophages are the key cells determining the host's response to Mycobacterium tuberculosis. The monocytes/macrophages phagocytize acid-bacilli. The effectiveness of this process depends on age, sex, genetic factors and the Fc and C receptors expression of monocytes [5-13].

The phagocytosis causes an increase in metabolic activity of cells, which express itself in an intensified consumption of oxygen, and activation of pentose cycle [8,14,15]. The results of the metabolic transformation is a partial reduction of oxygen and a release of a number of highly active chemicals, which can destroy bacteria [4,8,10,16-20].

An indirect marker of the consequences of the metabolic activity is the reduction of nitroblue tetrazolium (NBT) by monocytes [14,21,22]. The NBT test study in tuberculosis has

been rarely reported and the results were inconsistent [16--19,23,24]. To the best of our knowledge, this is the first such study on the monocyte capacity of tuberculosis patients of NBT reduction in an inactive tuberculosis.

Therefore, the aim of this study was to evaluate the ability of the NBT reduction by non- and BCG-stimulated monocytes isolated from peripheral blood of individuals with pulmonary tuberculosis before treatment, after two-month antituberculosis therapy and in an inactive stage of this disease.

Material and methods

Patients

With the approval of the Local Ethics Committee, fifty-nine unrelated patients with pulmonary tuberculosis: 33 patients before antituberculosis treatment and 26 with inactive stage of disease, were studied. In the group were 25 women and 34 men on the range age 20-73 years (mean: 41.1 ± 16.4). Patients with newly detected active TB were admitted at the Pulmonology Hospital in Sopot, Poland. A diagnosis of TB was established using standard clinical, radiographic, and bacteriological criteria [25]. The studied patients were at similar clinical stage and with similar localized disease on the initial chest radiographs (CXRs) (the infiltrates with cavitation in one or two lung zones). The diagnosis of TB was confirmed in all patients by demonstration of acid-fast bacilli (AFB) in sputum smears and by positive sputum culture of the M. tuberculosis strains. The positive PPD skin – test was the additional criterion including to the study group. After a two month-therapy (rifampin, isoniazid, ethambutol, pyrazinamide), 12 patients were examined again and none of them showed the clinical evidence of active pulmonary tuberculosis. Response to therapy was defined as clinical and radiologic improvement with disappearence of AFB in the sputum on smear examination within 2 months from the start of therapy. The remaining patients were lost to follow-up. The other group included 26 patients who had documented completion of antituberculosis treatment and negative sputum smears for AFB and culture results, follow-up ranged from 1 to 17 years (mean, 14 years) in the Outpatient Tuberculosis Departments in Gdańsk. The current clinical examination and the CXRs (the tuberculous fibrotic scarring with caverns in the affected lung zones) excluded an active stage of the disease. None of these individuals relapsed during the period of follow-up. None of the patients had a family history of TB or other related diseases.

Controls

A total of 24 age- and sex-matched, unrelated healthy volunteers were included in the study as the controls. The individuals were screened and recruited from our University staff. Based on detailed clinical history, controls did not have a history of TB. None of them exhibited attributes of active TB or other related diseases based on clinical examination.

Tuberculin skin test

All tuberculin tests were done by the Mantoux method using 2 TU of human PPD Rt_{23} with Tween® 80 (Statens Sevminstitut, Copenhagen). The tests were read at 48 to 72 hours after administration. Two transverse diameters of induration being measured to the nearest millimeter. Any reaction ≥10 mm was considered a positive test [2].

Mycobacteriology

All tests were performed using standard methods that did not change during the study period. Direct sputum smears were examined by light microscopy and by cultures on 7H11, Löwenstein-Jensen medium. All cultures were identified as catalase/peroxidase – INH-sensitive M. tuberculosis strains by standard test [25]. The niacin production test for M. tuberculosis was used. The first culture with positive result was routinely tested for drug susceptibility to first-line drugs administered to all patients. No other biologic differences were observed in the M. tuberculosis strains.

CXR

The initial and follow-up standard posteroanterior chest radiographs of the patients and controls were reviewed. The CXRs were evaluated by two physicians who were blinded to the clinical data of the study population. The disease extent on the initial CXR was based on the number of lung zones involved; each lung was considered to have three zones (upper, middle, and lower). Involvement of one or two zones was considered to be localized disease. Follow-up CXRs were classified as showing improvement, progression, or no change. The presence or absence of infiltrate and/or cavitation was noted.

Assays performed

Isolation of mononuclear cells from peripheral blood by Bøyum method [26]: 10 ml of peripheral blood was put into test-tubes containing 14 mg of disodium-EDTA and 1 ml of 6% Dextran 500 (Pharmacia). After mixing the tubes were put into thermotast (37°C) for 40 minutes. Next the mixture was centrifuged 700 rpm for 10 minutes. The sediment was left aside and the received supernant was centrifuged 4 400 rpm for 30 min to obtain low platelet plasma, and then it was added to the previously remained sediment. This mixture was layered on 3 ml Nycodenz (Nycomed Pharma, Batch 710294), placed in a silicon test-tube and centrifuged at 600 xg for 15 min. The forming interface and the 2 mm region with monocytes lying below it were gathered with pipette and afterwards were washed with PBS. Then they were led to the volume of 1 mm and received suspension of cells with 95% of monocytes. The viability of the isolated monocytes was tested by 1% of trypan blue. Monocytes were indetified by histochemical methods [27]. The presence of brown granules in cytoplasma was identified as possitive result of this reaction. The results were performed after classification of each of 100 accepted cells recorded to a degree of intensification of the reaction. The results were given as a sum of products of all numbers with positive reaction multiplied by the degree of the reaction.

Evaluation of the reduction abilities of NBT by non-stimulated monocytes [22]: 100 μl of suspension of monocytes ($1x10^6$/ml), 100 μl of human serum AB Rh^+, 100 μl of PBS and 50 μl of NBT (Fluka AG, Buch SG: 6 mg/ml) were added consequently to a plastic test-tube. The mixture was incubated (37°C) for 15 minutes. Then it was cooled (4°C) for 3 minutes and

Table 1. **The ability of the NBT reduction test by non-stimulated monocytes in the tested groups**

Tested groups	No.	Mean in % ± SD
Controls	24	26.1 ± 6
TB patients before therapy	33	39.3 ± 14*
TB patients after 2-months therapy	12	38.6 ± 9#
Individuals with inactive TB	26	29.4 ± 7**†

TB – pulmonary tuberculosis
* – p<0.001 for the controls vs TB before therapy
# – p<0.001 for the controls vs TB after 2-months therapy
** – p<0.01 for inactive TB vs TB before therapy
† – p<0.01 for inactive TB vs TB after 2-months therapy

Table 2. **The ability of the NBT reduction test by BCG-stimulated monocytes in the tested groups**

Tested groups	No.	Mean in %± SD
Controls	24	33.8±9
TB patients before therapy	33	44.5±12*
TB patients after 2-months therapy	12	44.3±7#
Individuals with inactive TB	26	34.8±4**†

TB – pulmonary tuberculosis
BCG – bacillus Calmette-Guerin
* – p<0.01 for the controls vs TB before therapy
# – p<0.01 for the controls vs TB after 2-months therapy
** – p<0.01 for inactive TB vs TB before therapy
† – p<0.001 for inactive vs TB after 2-months therapy

centrifuged at 700 rpm/5 minutes. A cell smear was prepared and stained according to Mäy-Grünwald-Giemsa staining were done. Counting of monocytes of at least containing precipitated formazane were performed under immersion.

Evaluation of the reduction abilities of NBT by BCG-stimulated monocytes [28]: 100 µl of suspension of monocytes ($1x10^6$/ml), 100 µl of human serum AB Rh+, 100 µl of PBS, 10 µl (0.05 mg) of BCG and 50 µl of NBT (6 mg/ml) were added consequently to a plastic test-tube. The rest of the procedure as above.

Statistical analysis

Statistical analysis was performed by means of STATISTICA for Windows v. 5.0 (StatSoft) software using the Student-t test for groups comparison. Statistical significance was defined as $p \leq 0.05$. Data are presented as mean ($\bar{x}$) ± SD for each individual tested parameter.

Results

The ability of NBT reduction by non-stimulated monocytes in the tested groups is presented in *Tab. 1*.

The average percentage of test positivity by monocytes was significantly higher in an active TB as compared with the controls ($p<0.001$). A two-month treatment no change of NBT reduction by monocytes in comparison to the values in TB patients before therapy, but the values were higher than in the controls ($p<0.05$). In an inactive TB, the ability of NBT reduction was comparable to healthy people.

The ability of NBT reduction by BCG-stimulated monocytes in tested groups is presented in *Tab. 2*.

The average percentage of positivity of stimulated NBT reduction test by monocytes was significantly higher in an active TB vs the controls ($p<0.01$). After two-month treatment the values were not changed. In an inactive TB, the ability of NBT reduction by BCG stimulated monocytes was similar like in the group of healthy people.

The comparison of the ability of NBT reduction by non-stimulated and BCG-stimulated monocytes in tested groups is presented in *Fig. 1*.

In the group of patients with active TB, the values of the NBT reduction tests by non-stimulated and BCG-stimulated monocytes remain at the same level. A two-month treatment did not change the ability of spontaneous and stimulated NBT reduction in comparison to the values in TB patients before therapy. However, in the controls ($p<0.01$) and in inactive TB ($p<0.05$) the values of the NBT test by BCG-stimulated monocytes significantly increase as compared with the ability of reduction by non-stimulated monocytes ($p<0.01$; $p<0.05$, respectively).

Discussion

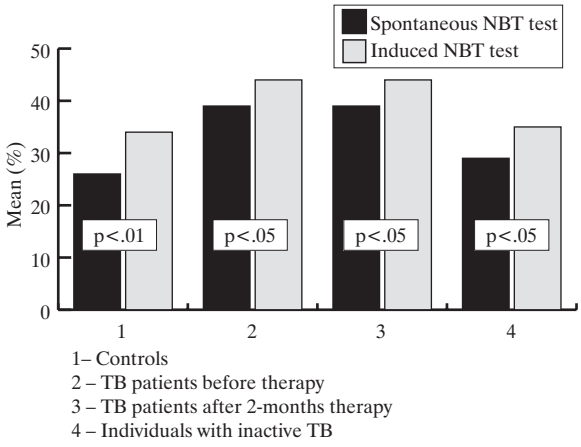
The nitroblue tetrazolium (NBT) test is an indirect marker of the oxygen-depend bactericidal activity of the phagocytes [4,8,10,14,20].

NBT is a dye with low reduction potential and performs intensively stained products–formazanes. NBT is easily phagotized by cells and is reduced to formazane inside mitochondrium.

The spontaneous NBT test use for screening of metabolic activity of granulocytes and/or monocytes. The induced NBT test assess the functional abilities of phagocytizing cells. The positive results of NBT reduction test show the percentage of stimulated cells by bacterial products phagocytes and occurrence of infection. The false positive results of NBT test exist during bacterial infection with presence of large necrosis or in limphoblastic leukaemia. False negative are present in myeloma [22].

The NBT reduction test and its modifications are useful for detection and differention of bacterial diseases. The NBT test on the granulocytes and monocytes can be used for the indirect evaluation of their bactericidal potency in non-specific infections but there are few inconsistent observations in tuberculosis [16-19,23,24,29]. Gracheva et al. [19] and Kaminskaja et al. [16,18,19,29] demonstrated the increasing insensitivity of the NBT reduction, whereas Garbiński et al. [23] and Shatrov et al. [24] revealed decreased abilities of nitroblue tetrazolium reduction by granulocytes, monocytes in an active tuberculosis. These cells, though capable of bactericidal action, do not react to stimulation Mycobacterium tuberculosis after 4 weeks of beginning of TB therapy [19,29]. To the best of our knowledge,

Figure 1. **The comparsion of the abilities of NBT reduction by non-stimulated nad BCG-stimulated monocytes in tested groups**



no report about the evaluation of the abilities of the NBT reduction by monocytes in an inactive tuberculosis.

According to Gracheva et al. [19] and Kaminskaja et al. [29], our results show the increased NBT reduction properties of monocytes both in an active tuberculosis and after two-month treatment. This increase of the ability of reduced of nitroblue tetrazolium can be a result of involvement of the oxygen-dependent mechanism of bactericidal activity of monocytes. The positive results of NBT tests after two-month treatment may suggest the presence of mycobacterial stimulation of monocytes or/and occurrence of necrosis in the affected tissue. The analysis of the spontaneous and induced NBT suggests a decreased of monocytes energetic reserve during additional stimulation of Mycobacterium tuberculosis in an active TB. However, monocytes isolated from healthy people and individuals with an inactive TB had greater abilities of bactericidal reduction after BCG stimulation in vitro.

Conclusions

These results of the spontaneous and induced NBT tests adequately reflect the status of the host's specific reactivity during tuberculosis and can be simple, cheep and useful for a monitoring of antituberculosis treatment.

Acknowledgement

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Laminin, Her2/neu and Ki-67 as prognostic factors in non-small cell lung cancer

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Abstract

Purpose: **The aim of this study was to determine the values of Ki-67 antigen, oncoprotein Her2/neu and laminin, as prognostic and predictive factors in NSCLC, and on the basis of these markers to create a prognostic model which would make it possible to identify patients with a high risk of disease recurrence.**

Material and methods: **The material for the study came from 64 patients with NSCLC, who underwent surgery in Dolnośląskie Centrum Gruźlicy i Chorób Płuc i 1996--2000, and subsequently were given radiation therapy in Dolnośląskie Centrum Onkologii.**

Results: **Among the markers researched, a high level of (intracellular) laminin in carcinoma cells was found to be an unfavourable prognostic factor. Also, another group of patients with an overexpression of oncoprotein Her2/neu were found to have a poorer prognosis, although the influence of proliferative index Ki-67 on patient survival could not be explained. The prognostic model LAMHER, which was defined on the basis of the intracellular laminin level and expression HER2/neu, enables the identification of a group of patients with a high risk of disease recurrence. In a multidimensional analysis of the "classification tree", it was found that patients with the highest risk of disease recurrence were those with LAMHER = 1 (overexpression Her2/neu and/or a high level of intracellular laminin), and patients with LAMHER = 0 but a Fractionation Dilution Factor higher than 1.57.**

Conclusions: **The conclusion of this study is that multiple molecular marker testing is necessary to detect an independent prognostic impact on survival and is therefore superior to single marker testing. Based on LAMHER testing, two groups of patients could be defined: a low-risk group (LAMHER = 0) and a high-risk group (LAMHER = 1) for failure of standarized treatment.**

Key words: laminin, Her2/neu, Ki-67, non-small cell lung cancer, postoperative radiotherapy.

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Introduction

Lung cancer was the most common cause of cancer deaths in 1996 in the region of Lower Silesia, Poland. Lung cancer composed 10.8% of all malignancies among women, second only to breast cancer. Among men it was the most common type of cancer, at 28.1% among all cancer morbidity. Five-year survival among men reached 7%, and among women 12.1% [1]. Surgery remains the basic treatment modality and offers the best chance to cure patients with NSCLC. However, less than 1/3 of these patients are suitable for this kind of treatment; the results of their treatment are not the best, because even if it is possible to conduct radical surgery most patients are not free of the disease. According to the Jassem study, five-year survival following surgery was in stage IA – 66%, IB – 53%, IIB – 30%, and IIIA – 15% [2]. These not so optimistic results have generated a search for new prognostic factors which would allow us to define groups of patients who require more intensive anticancer therapy.

Material and methods

Material for the study came from 64 patients with primary non-small cell lung cancer, who were operated on in DCGiChP and postoperatively underwent radiation therapy in DCO from 1996 until 2000. Some of them had also postoperative chemotherapy. Histopathologic types of

***Table 1*. Histopathologic types of primary tumours and treatment modalities**

		Histopathology			
		squamous ca.	**adenoca.**	**large cell ca.**	**In total**
No of patients		24	23	17	64
Type of operation	wegde resection	0	1	0	1
	lobectomy	10	17	10	37
	bilobectomy	2	2	0	4
	pulmonectomy	12	3	7	22
No of chemotherapy cycles	0	14	9	5	28
	1-2	2	3	7	12
	3	5	9	5	19
	4-6	3	2	0	5
Fractionation of postoperative radiotherapy	df=2Gy	21	14	13	48
	df=2.66Gy	3	9	4	16

***Table 2*. Disease Free Survival (DFS) and Overall Survival (OS) in weeks from the date of the operation**

	Women	**Men**	**Medium (weeks)**		**Minimum (weeks)**		**Maximum (weeks)**		**Standard deviation**	
			DFS	**OS**	**DFS**	**OS**	**DFS**	**OS**	**DFS**	**OS**
Healthy	6	14	182	182	104	104	285	285	53	53
Distant progression	6	13	59	105	10	21	155	223	31.4	51
Loco-regional progression	2	7	95	129	44	62	175	209	52.4	54
Loco-regional and distant progression	1	5	68	98	27	56	143	153	42	38
Another cancer	0	2	96	141	54	92	138	190	59.3	69
Death without cancer progression	0	8	85	85	32	32	213	213	65	65
In total:	**15**	**49**	**107**	**130**	**10**	**21**	**285**	**285**	**69.5**	**63**

primary tumours and treatment modalities are indicated in *Tab. 1*. Immunohistochemical analyses were performed on paraffin blocks of resected lung tissue from 64 patients. Primary monoclonal antibodies to Ki-67, Her2/neu and laminin (DAKO Rabbit Anti Human Ki-67 Antigen N 1574 LSAB /control slides N 1574/, Rabbit Anti Human c-cerbB-2 Oncoprotein N 1629 LSAB /control slides N 1629/ Monoclonal Mouse Anri Human Laminin klon 4C7 Isotype IgG2a, kappa) were used. All immunohistochemical data were assessed twice for each patient, without prior knowledge of the patient's outcome. Proliferation index Ki-67 was determined by scoring the percentage of malignant cells with positive nuclear staining in five microscope fields, using the microscope Olympus BX – 50 and the computer program MultiScanBase 08.98. Her2/neu expression was estimated according to a semiquantitative four-stage grading system. Laminin expression was also evaluated using the semiquantitative system. Separately, on a three-grade scale the percentage of the cells with laminin expression (0%, <50%, >50% cells) was estimated, and separately on a four-grade scale was estimated the intensity of intracellular laminin staining (0-3 grade). The extracellular laminin expression was evaluated using a semiquantitative four-stage grading system (0-3 grade). Overall cancer-specific survival was defined as the period from the date of operation to the date of cancer-death. An observation was made at the last follow-up to determine if the patient was either alive or had died of a cause other than NSCLC. Disease Free Survival was defined similarly. Kaplan--Meier curves were calculated for each variable. The log-rank test and the Cox proportional hazards model were used to examine the relationship between cancer-specific survival, disease-free survival and various potential prognostic factors. The level of significance was set at $p<0.05$. The association of all markers with clinicopathological parameters was evaluated using the Chi-Square Test. Statistical analysis was performed using STATISTICA ver. 6. The analysis of classification trees, one of the most popular techniques in data mining, was performed using STATISTICA ver. 6.

Results

The results of 64 patients treated for NSCLC were analysed statistically. We examined the association between the following parameters and their influence on survival and disease-free survival-gender, age, type of operation, radicality of operation, pTNM, histologic subtype, grade of histologic malignancy, number of chemotherapy cycles, total dose of radiotherapy, dose intensity, fractionation dilution, proliferation index Ki-67, Her2/neu expression, intracellular laminin expression, and extracellular laminin expression.

Tab. 2 shows the mean time of DFS and OS. Index Ki-67 was estimated for 47 patients. The mean index Ki-67 was 28.6% (range 0.5 to 90%). The only weak correlation was between high expression Her2/neu and low index Ki-67, without statistical power. There was no difference in DFS and OS between groups of patients with index Ki-67 >25% and <25%.

Figure 1. **Disease free survival for patients with intracellular laminin in more than 50% and less than 50% of the cells (p=0.02)**

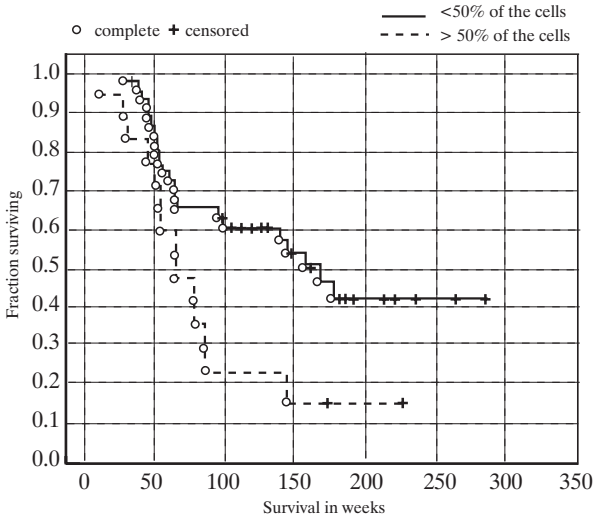


Figure 2. **Overall survival for patients with intracellular laminin in more than 50% and less than 50% of the cells (p=0.008)**

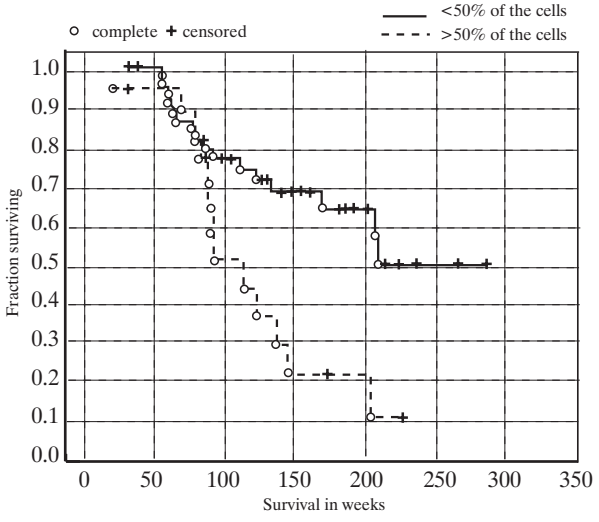


Figure 3. **Disease free survival for patients with the most intensive reaction against intracellular laminin and the rest of patients (p=0.02)**

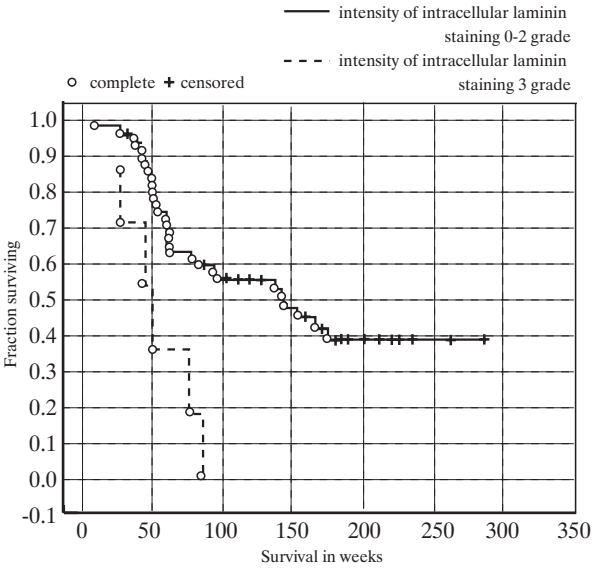
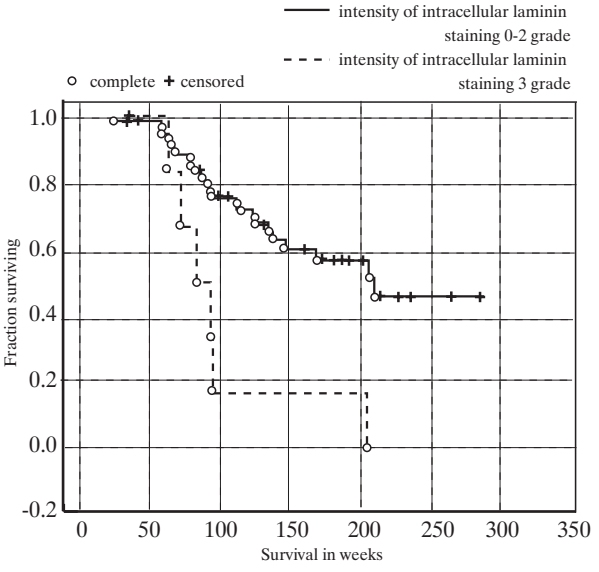


Figure 4. **Overall survival for patients with the most intensive reaction against intracellular laminin and the rest of patients (p=0.01)**



Her2/neu expression was evaluated for 64 patients, and was estimated as 0 for 21 (32.8%) patients, as 1 degree for 23 (35%) patients, as 2 degrees for 13 (20%) patients, and as 3 degrees for 7 (10%) patients. There wasn't any significant dependence between Her2/neu expression and other parameters.

DFS (*Fig. 1*) and OS (*Fig. 2*) were found to be statistically diminished among patients with laminin expression in more than 50% cancer cells. We found also to be statistically significant the difference in DFS (*Fig. 3*) and OS (*Fig. 4*) between patients with the most intensive reaction against intracellular laminin and the rest of the patients. There were no significant difference in DFS and OS on the other levels of extracellular laminin.

To identify groups of patients with poorer prognosis, we used tissue markers whose presence leads to a decrease of DFS – it was intracellular laminin and Her2/neu – whose influence on DFS was close to statistical significance. From among the 64 patients we constituted a group in which at least one of the chosen markers was on the highest level (Her2/neu overexpression or/and laminin in more than 50% cells or/and intracellular laminin staining intensity =3); and to simplify description in a subsequent analysis we introduced the term LAMHER. Comparison of curves of DFS (*Fig. 5*) and OS (*Fig. 6*) between groups of patients with at least one of the chosen markers on the highest level (LAMHER = 1) and the

***Figure 5*. Disease free survival for patients with LAMHER=1 and LAMHER=0 (p=0.0014)**

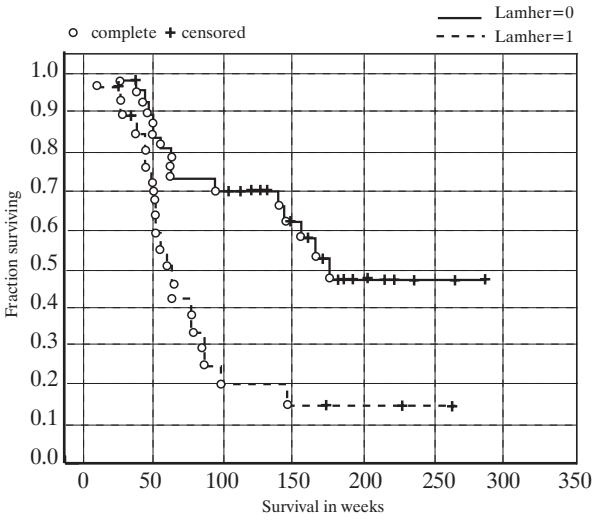


***Figure 6*. Overall survival for patients with LAMHER=1 and LAMHER=0 (p=0.00017)**

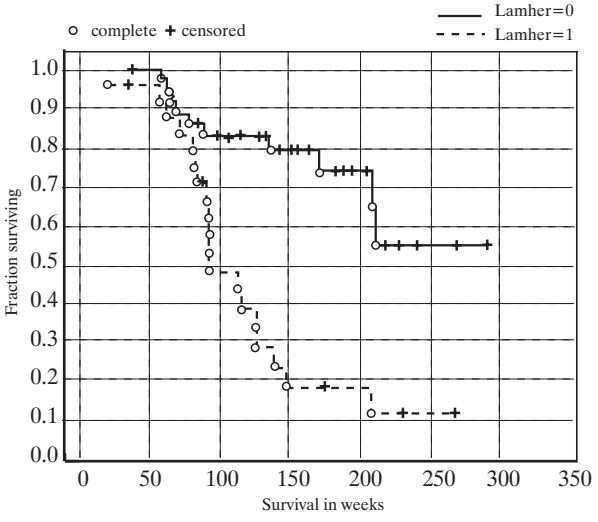
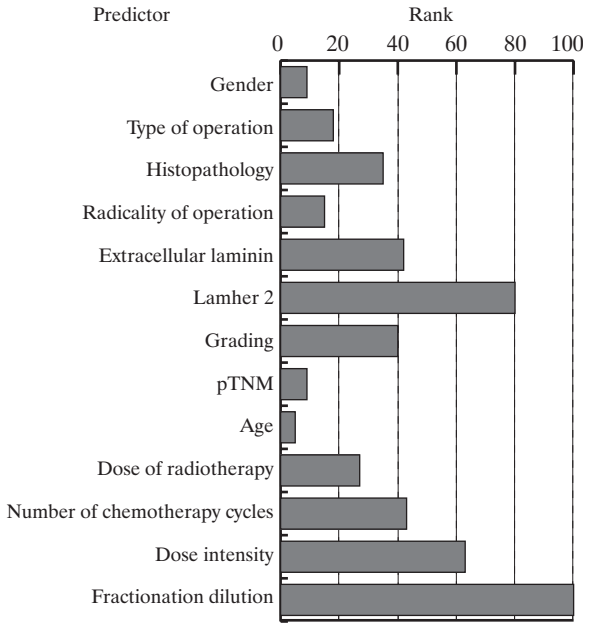


***Figure 7*. Rank of the parameters as predictive factors for reccurrence of the disease scale from 0 (low value) to 100 (high value)**



rest of studied patients (LAMHER = 0) showed a statistically significant difference.

Multivariate analyses was performed with the Cox proportional hazards regression model. From among the factors, only the level of the LAMHER factor was found to be the only independent risk factor (p=0.001, RR = 4.08).

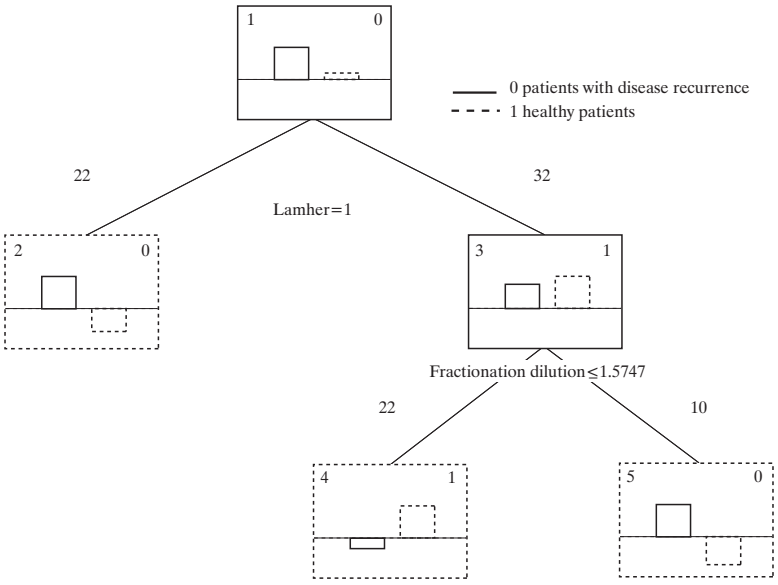
We used a multidimensional exploratory technique – analysis of classification trees – to find such predictive factors which would enable one, in the most exact and easiest way, to prognosticate recurrence of the disease. Analysis was based on observation of 54 patients; we excluded patients who died without cancer progression. *Fig. 7* shows the parameters taken into consideration in this analysis, and their rank as predictive factors. The most unfavourable prognostic factors according to a computerised algorithm were LAMHER and Fractionation Dilution (FD = overall time of radiotherapy/number of fractions radiotherapy). The scheme of the classification tree simply shows that the chance of curing patients with LAMHER equals 1 is close to 0, and for this group of patients the significance of the other factors is almost inessential. In the group with LAMHER equals 0 was the prevalence of patients without disease recurrence. Application of the Fractionation Dilution factor in this group enables one to distinguish between patients with better (FD≤1.57) and worse (FD>1.57) prognosis. Using this rule in our study, 10 of 54 patients were wrongly classified, six as healthy while they had recurrence of the disease, and four patients as ones with disease recurrence while in fact they were healthy. Global costs of faulty classification after the cross-matching test was 0.33.

Discussion

We designed this study to test the hypothesis that Her2/neu expression, the level of intracellular laminin and index Ki-67 by themselves or in combination are of prognostic importance in patients with resected NSCLC followed by postoperative radiotherapy. The markers in this study effect the malignant transformation and metastatic process through three oncogenic mechanisms: cell growth – Her2/neu, cell cycle regulation – Ki-67 and invasiveness – laminin.

We did not find a statistically significant difference between low and high proliferative indices. The relationship between Ki-67 expression and prognosis in NSCLC is still controversial.

Figure 8. **The tree classifying patients to high and low risk group for recurrence of the disease On the left side, there are histograms of the patients fulfilling the above-mentioned condition. Figures in the upper right corner mean which group of patients is in the prevalence**



However, most of the studies suggest decreased survival among patients with high Ki-67 expression [3-11]. In 2001 Ramnath showed limited value for the Ki-67 index, and concluded that Ki-67 index confidence intervals are large and weakly correlate with survival. He corroborated the importance of the Ki-67 index as a prognostic factor in patients with NSCLC is marginal [12].

Although the Kaplan-Meier survival analysis did not demonstrate the negative prognostic value of Her2/neu, there was a trend for poorer overall survival and disease free survival in patients with Her2/neu overexpression. However, a large number of patients would be necessary to prove a statistically significant effect of Her2/neu overexpression, more particularly as only 10% of our patients showed its overexpression. The major problem in comparing studies of Her2/neu expression in NSCLC is the enormous variations in frequencies of NSCLC tumours scored positive for Her2/neu, due to the application of different techniques and antibodies [13-27]. As a consequence different results were reported concerning the impact of Her2/neu overexpression on survival in NSCLC [16,18,21--23,28]. Most of the studies confirming a statistically important decrease of survival in patients with Her2/neu overexpression was performed on populations of patients with stage I NSCLC [3,17,18,24,29]. In the case of higher stage NSCLC, impact of the single marker on survival could be less distinct.

The factor with the strongest independent prognostic value was laminin. Kaplan-Meier overall and disease free survival analysis demonstrated the negative prognostic value of the intensive intracellular laminin expression and also the presence of laminin in more than 50% of cells. We used monoclonal antibody 4C7, which was acknowledged to be specific for the laminin a1 chain, but the latest studies have proved that it recognised rather the laminin a5 chain [30,31]. Most of the studies concerning the relation between laminin and cancer progression were based on laminin 5 (a3, b3, g2). In the case of this isoform, there was a dependence between laminin chains accumulation and malignant progression of the cancer [32-39]. There are very few publications concerning laminin in NSCLC [40-42].

To create a prognostic model we used molecular markers of two oncogenic mechanisms that have an influence on Disease Free Survival, namely intracellular laminin and Her2/neu, and we called these markers LAMHER. In the investigated group, only one patient had both Her2/neu overexpression and the highest level of intracellular laminin. We found a statistically significant difference between patients with LAMHER = 1 and the rest of the population in DFS ($p = 0.0014$) and OS ($p = 0.00017$). In the Cox analysis, the factor with the strongest independent prognostic value was LAMHER ($p<0.001$, RR = 4.08).

In the last years, a few studies were published in which the authors created biological risk models based on multiple molecular markers and showed that survival depends on the "dose" of cumulative unfavourable prognostic factors [3,17,24,29,43]. The conclusion of this study is that multiple molecular marker testing is necessary to detect an independent prognostic impact on survival and is therefore superior to single marker testing. Based on LAMHER testing, two groups of patients could be defined: a low-risk group (LAMHER = 0) and a high-risk group (LAMHER = 1) for failure of standarized treatment.

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Loss of heterozygosity in laryngeal cancer

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Abstract

Purpose: Head and neck cancers account for about 6% of all human cancers. Molecular changes leading to the disease development and progression still remain not fully explained. Examination of loss of heterozygosity (allelic loss, LOH) using the specific microsatellite markers is a method of choice in assessing tumour suppressor genes (TSGs) localisation in human genome.

Material and methods: The study was performed in a group of 46 male patients, aged 42-77 years. Forty three patients underwent total laryngectomy with lymph nodectomy, two patients – chordectomy and one patient – partial laryngectomy.

Tumour tissue specimens and reference peripheral blood samples were obtained during surgical resections. Standard methods were used for DNA isolation. Fluorescent multiplex PCR was used to amplify microsatellite loci included in commercially available human identification kits.

Results: LOH was found at the following loci: BAT26, D3S1358, FGA, CSF1PO, D5S818, D8S1179, VWA, D13S317, D18S51. The highest LOH frequency was found in the tumor samples where the neighbouring cervical lymph nodes were affected but the incidence of LOH at BAT26 was statistically insignificant ($p = 0.07$).

Conclusions: High incidence of LOH is considered an unfavourable prognostic factor accompanying an aggressive nature of the tumour and indicating an involvement of certain genome regions in cancerogenesis. In head and neck cancers LOH was found on the following chromosomes: 3p, 5q, 8p, 9p, 9q, 11q, 17p, 17q, 18p, 18q.

Key words: laryngeal cancer, loss of heterozygosity.

Introduction

Head and neck cancers account for about 6% of all human cancers. They are more common in men than in women (the ratio about 3 to 1). Cytogenetic changes, including MYC or RAS proto-oncogene inactivation and inactivation of tumour suppressor genes (TSGs) of cancerous transformation are discussed as etiological factors. On the other hand, suppressor gene products may inhibit cancerous phenotype. Molecular changes leading to the disease development and progression still remain not fully explained. Examination of loss of heterozygosity (allelic loss, LOH) using the specific microsatellite markers is a method of choice in assessing TSGs localisation in human genome. More detailed understanding of these disturbances will help to establish possible prognostic factors in the disease treatment. LOH, or loss of normal allele in heterozygotic locus, may result from different mechanisms, including chromosomal deletion, mitotic recombination (MR), gene conversion, point mutation, or intragenic allele inactivation [1]. Consequently, the locus may become homozygous due to mitotic recombination, gene conversion or chromosome loss with reduplication, hemizygous due to deletion or chromosome loss, complex heterozygote due to introduction of another point mutation at the locus, or it may remain heterozygous (with relation to nucleotide sequence), if one allele is inactivated intragenically [2].

High incidence of LOH is considered an unfavourable prognostic factor accompanying aggressive nature of the tumour and indicating involvement of certain genome regions in cancerogenesis. In head and neck cancers LOH was found

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Table 1. **Chromosomal distribution of LOH frequency in laryngeal cancer**

Marker	Chromosomal localisation	LOH frequency (%)
BAT26	2p16.3-q21	19/45 (42%)
D3S1358	3p	6/45 (13%)
FGA	4q28	2/45 (4%)
CSF1PO	5q33.3-34	8/45 (18%)
D5S818	5q21-q31	5/45 (11%)
D8S1179	8q	1/45 (2%)
VWA	12p12	3/45 (7%)
D13S317	13q22-q31	4/45 (9%)
D18S51	18q21	7/45 (16%)

Figure 2. **LOH incidence in laryngeal cancer**

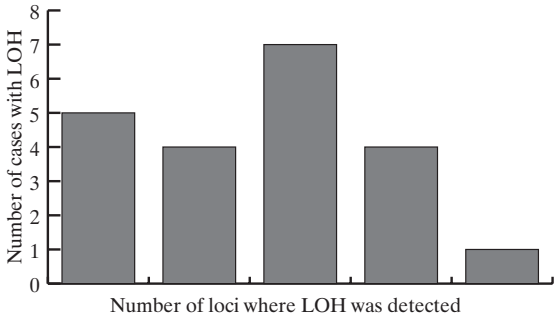
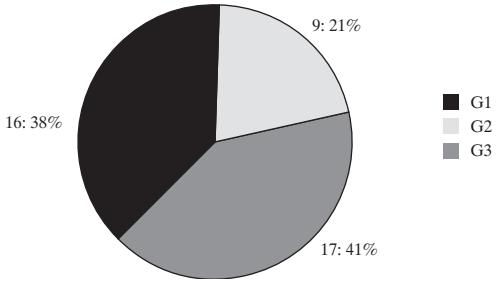


Figure 1. **LOH incidence and histopathological grade (G1, G2, G3): number of cases; % of cases**



on the following chromosomes: 3p, 5q, 8p, 9p, 9q, 11q, 17p, 17q, 18p, 18q. Changes in other DNA regions are also possible [3].

Material and methods

The study was performed in a group of 46 male patients, aged 42-77 years. Forty three patients underwent total laryngectomy with lymph nodectomy, two patients – chordectomy and one patient – partial laryngectomy. Tumour tissue specimens and reference peripheral blood samples were obtained during surgical resections. Standard methods were used for DNA isolation. Additional microcolumn purification was performed when necessary. Fluorescent multiplex PCR was used to amplify microsatellite loci included in commercially available human identification kits: AmpFlSTR Profiler and AmpFlSTR SGM Plus (Applied Biosystems, USA) according to the manufacturer's recommendations. Genotyping was performed in 310 ABI Prism Genetic Analyzer (Applied Biosystems, USA) using the GeneScan Analysis v3.1 and Genotyper v2.5 software. The examined microsatellite markers and targeting genes are presented in *Tab. 1*. Amplification of the BAT26 locus was performed in a separate reaction using PCR Core Kit (Qiagen, USA) and commercially synthesized primers of the following sequences: forward primer 5'-tga cta ctt ttg act tca gcc, reverse primer 5'- aac cat tca aca ttt tta acc. PCR conditions were as follows: initial denaturation at 94°C - 1 min, 35 cycles of 94°C - 30 s, 49°C - 40 s and 72°C - 40 s with the final elongation at 72°C for 10 min. PCR products were separated using horizontal non-denaturing PAGE in a discontinuous buffer system and silver stained. The analyses were performed twice, so the results were highly reproducible. LOH was defined as a decrease (at least 50%) in peak height of an allele compared with that of the other determined after comparison of normal and pathologic DNA. Amplicons were sized according to 100 bp ladder (Gibco, BRL). Frequency of LOH was calculated for respective samples. Results were analyzed using a statistical analysis program commonly applied in biomedical assays (t-Student test for pairs of variables).

Results

On histopathologic specimens, G1 grade cancers were diagnosed in 9 patients (21%), G2 grade cancers in 17 patients (41%) and G3 grade cancers in 16 patients (38%) (*Fig. 1*). Lymph node metastases were found in 16/45 cases, with G1 in 2 patients, G2 in 5 patients and G3 in 9 patients. LOH was detected in 31% cases: one patient (2%) displayed LOH at 5 loci, 4 patients (9%) at 4 loci, 7 patients (16%) at 3 loci, 4 (9%) patients at 2 loci, 5 patients (11%) at 1 locus (*Fig. 2*). LOH frequency was statistically significant in high G grade cancers ($P=0.0034$).

Overall, 39 (87%) of 45 patients displayed LOH at the following loci: BAT26 – 19/45 (42%), D3S1358 – 6/45 (13%), FGA – 2/45 (4%), CSF1PO – 8/45 (18%), D5S818 – 5/45 (11%), D8S1179 – 1/45 (2%), VWA – 3/45 (7%), D13S317 – 4/45 (9%), D18S51 – 7/45 (16%). No LOH was found at the other microsatellite markers. No evidence of LOH was found at the other markers.

The highest LOH frequency was found in the tumor samples where the neighbouring cervical lymph nodes were affected. In 8/16 histopathologically verified cases with lymph node metastases LOH was found at BAT26 and at other studied STR loci, in other two cases – only at BAT26. The incidence of LOH at BAT26 was statistically insignificant ($p=0.07$). In 5/29 patients with non-involved cervical lymph nodes LOH was present at BAT26 and the particular chromosomal localisation (*Tab. 1*).

Discussion

In the present study, LOH was found in 39 out of 45 cases (87%). Comparable data was obtained by El-Naggar et al., whereas Jin et al. found no statistically significant association between major karyotypic features and histological differentiation or TNM stage in the laryngeal cancer [4,5]. Microsatellite markers are commonly used in the analysis of allelic imbalance. Presence of LOH at numerous loci in tumor specimens may contribute to the development of a model of cancerogenous progression [6]. Application of microsatellite loci as genetic markers is common due to their abundance in the genome, extreme polymorphism and amplification by the PCR reaction. In 1996 Califano et al. proposed a model of genetic changes in head and neck squamous cell epitheliomas, which assumes positive correlation of LOH and histological grade, spanning from mild hyperplasia to invasive cancer [7] .Veltman et al. reported LOH at 18q21 in pre-malignant laryngeal lesions, which suggests involvement of this chromosomal region in the laryngeal carcinogenesis [8].

El-Naggar et al. analysed LOH in 20 patients suffering from head and neck squamous cell epithelioma [3]. According to these authors, in non-invasive cancers LOH was observed on the following chromosomes: 9p (28%), 9q and 18q (10%), 11q and 17p (7%), 3p and 18p (5%). High incidence of LOH in invasive cancers was observed on chromosomes: 9p (72%), 8p (53%), 3p (47%), 9q (35%) and 11q (33%). LOH was also correlated with DNA aneuploidy, high grading and low histologic differentiation [3]. In the present study the authors failed to obtain statistically significant correlation between LOH frequency and metastases to the lymph nodes.

Conclusions

1. Our findings suggest positive correlation between LOH incidence and G grade in laryngeal cancers.
2. No statistically significant correlation was found between LOH frequency and metastases to the cervical lymph nodes.

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Effects of antidepressant mirtazapine on fibromyalgia symptoms

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Abstract

Purpose: **Fibromyalgia syndrome (FS) is a form of non-articular rheumatism. The main criteria are the widespread musculoskeletal pain and tender points at multiple characteristic sites which are associated with several vegetative and functional symptoms. Depression is the most frequent psychiatric concomitant of FS. Etiology is unknown, connection between disturbances of serotonin metabolism and pathogenesis is postulated. Pharmacological therapy with analgetic and nonsteroidal antiinflammatory drugs is not very effective. Positive effects were reported in some patients treated with antidepressant drugs, especially serotonergic agents.**

Material and methods: **In the study a novel antidepressant drug mirtazapine was used characterized by selective blockade of 5-HT2 and 5-HT3 receptors. In an open trial participated 29 patients with FS, who met 1990 ACR criteria for fibromyalgia. All were treated with mirtazapine for 6 weeks. Intensity of pain, sleep disturbances, fatigue and other symptoms were measured using visuale analogue scale, severity of depression was evaluated with HDRS and BDI.**

Results: **An open trial completed 26 patients, the majority of them experienced a clinical improvement at the end of the study as a consequence of ≥40% reduced intensity of fibromyalgia symptoms as well as reduced severity of depression. The significant correlation between reduction in depression after 6 weeks of mirtazapine treatment with the reduction on all four main symptoms of FS suggests a common pathophysiology of depression and symptoms of fibromyalgia. The data thus far obtained indicate the blockade of 5-HT2 and 5-HT3 receptors with mirtazapine as an effective and promising method in FS.**

Conclusions: **Further double-blind placebo-controlled study are required to confirm our results.**

Key words: fibromyalgia, blockade of 5-HT2 and 5-HT3 receptors, mirtazapine.

Introduction

Fibromyalgia syndrome (FS) is a form of non-articular rheumatism. Widespread musculoskeletal pain is the dominant symptom, and tender points at multiple characteristic sites can be demonstrated in most instances [1,2]. FS is frequently associated with chronic fatigue, poor sleep, irritable bowel, headaches, dysmenorrhea and other vegetative symptoms [3-5]. All studies confirm the female preponderance as well as the chronicity of symptoms in this syndrome [6,7]. Muscle biopses reveal no inflammatory processes and basic laboratory tests are normal [8,9]. Depression is the most frequent psychiatric concomitant of FS and observed association between FS and depression has been considered in several ways: is FS a variant of depression (somatized depression) or are the symptoms of depression secondary to disturbances in the process of coping with chronic and painful disease? Another possibility is that depression and FS share common aspects of their pathophysiology [10,11].

Several studies have shown that central serotonin abnormality may be connected with an increased pain sensitivity, sleep disturbance and depression [12]. Because these symptoms are prominent in FS, an association between FS and disturbed serotonin (5-HT) metabolism has been postulated [13]. There is some evidence supporting this hypothesis such as showing a decrease in both serum concentration of 5-HT and

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plasma concentration of tryptophan (5-HT precursor) in FS. A relationship exists between the 5-HT serum concentration and the intensity of pain as well as the number of specific "tender points" [14-16]. A presence of antibodies against 5-HT in patients with FS has been also demonstrated [17,18].

Pharmacological therapy of FS is mostly unsatisfactory. Analgetic agents and nonsteroidal antiinflammatory drugs are not very effective. On the other hand, a favorable therapeutic response was observed to antidepressant drugs. Positive effects were reported in some FS patients treated with tricyclic antidepressants – imipramine and amitriptyline [19,20], with selective serotonin reuptake inhibitors – citalopram and fluoxetine [21,22], and also with the novel antidepressant venlafaxine [23]. Influencing serotonergic system makes an important element of mechanism of each of these drugs. A recent meta-analysis of fibromyalgia treatment with antidepressant drugs suggests a significant effect of such treatment on sleep, fatigue, pain and well-being of patients. However, it is not clear whether this effect is related to antidepressant action [24,25].

Apart from antidepressant drugs, some effect in FS was also observed with serotonergic agents, e.g. selective blockers of serotonergic receptors. In the study with ketanserine, the drug blocking serotonin 5-HT2 receptors, it was found that quality of sleep in FS was improved [26]. Also, a therapy with tropisetron or ondansetron, the selective 5-HT3 receptor antagonists reduced pain intensity in about half of FS patients whereas no change in pain level was seen in the other half. The lack of uniform pattern of responsiveness to these agents may suggest a heterogeneity of pathogenic background in relation to serotonergic system in FS patients [27-29].

Mirtazapine is a novel antidepressant drug characterized, among others, by selective blockade of both 5-HT2 and 5-HT3 receptors. In view of previous moderately promising studies with selective serotonin receptor antagonists, we hypothesized that this drug may be useful in the treatment of fibromyalgia. In this study we present the results of an open trial of mirtazapine in FS patients.

Materials and methods

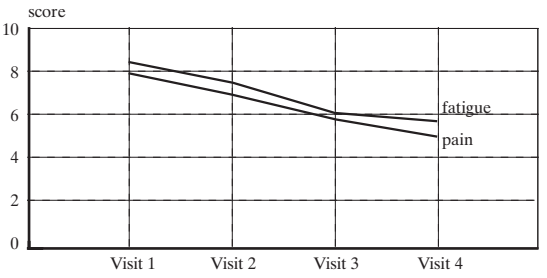
Subjects

The study group consisted of 29 patients with FS (25 female and 4 male) with mean age 45.6 years (range 20-64). All patients met the 1990 American College of Rheumatology diagnostic criteria for FS [30]. Each patient underwent routine clinical examination, radiographic and laboratory investigation to establish the diagnosis (excluding other inflammatory, rheumatic, metabolic, endocrine disease associated with muscle pain). They all gave written consent to the study which was also approved by the Ethics Committee, University of Medical Sciences, Poznań.

Procedure

The patients were free from all antidepressant medication for at least 7 days before the administration of mirtazapine. Throughout the study, no other drugs were permitted with the exception of paracetamol, if required by the patient, but not exceeding 2 g/day. The patients were treated with mirtazapine for 6 weeks (42 days), the first week with 15 mg in the evening, the next 5 weeks the dose could be increased to 30 mg in the evening. All patients were examined by the rheumatologist and psychiatrist before enrolling into the study (visit 1), after 7 days (visit 2), after 21 days (visit 3) and after 42 days (visit 4).

Figure 1. **Intensity of pain and fatigue in the courses of mirtazapine treatment. (VAS score)**



Assessment

Intensity of pain, sleep disturbances, fatigue and other symptoms: cold extremities, dryness of mouth, perfuse sweating, dizziness, gastric problems, headache or migraine, irregular breathing, arrhythmia, paresthesia and urinary urgency was measured using visual analogue scale (VAS) where 0=no symptom and 10=extreme intensity of symptoms [31]. The duration of morning stiffness was measured in minutes.

The severity of depression was measured using the Hamilton Depression Rating Scale (HDRS) – 17 item version [32] and Beck Depression Inventory [33].

Patient was considered improved, if he/she obtained the reduction of ≥40% of the following main symptoms: pain, fatigue, sleep disturbances and depression.

Statistics

For the statistical analysis, the Wilcoxon-Test, the Mann-Whithney U Test and ANOVA procedure were used.

Results

Among 26 patients who completed an open trial with mirtazapine, the majority experienced a clinical improvement at the end of the study as a consequence of ≥40% reduced intensity of fibromyalgia symptoms. In 13 patients (50%), the reduction of pain and fatigue level was observed. The decrease of intensity of these symptoms in whole group of patients with subsequent visits is depicted in *Fig. 1*.

The improvement of the sleep quality with significant reduction on VAS was noted in 19 patients (73%), what was also supported by the decrease of intensity on HDRS sleep subscale (items 4-6). This is shown in *Fig. 2*.

The reduced severity of depressive symptoms ≥40% on HDRS scale was observed in 18 patients (69%) and on BDI scale in 16 subjects (61%). The mean intensity of depressive symptoms during subsequent visits in shown in *Fig. 3*.

Figure 2. **Sleep disturbances in the course of mirtazapine treatment (VAS score and HDRS sleep subscale)**

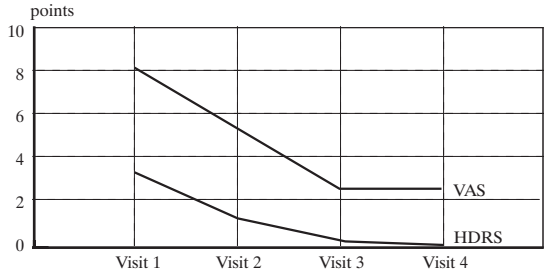


Figure 3. **Intensity of depression (HDRS and BDI scores) in the course of mirtazapine treatment**

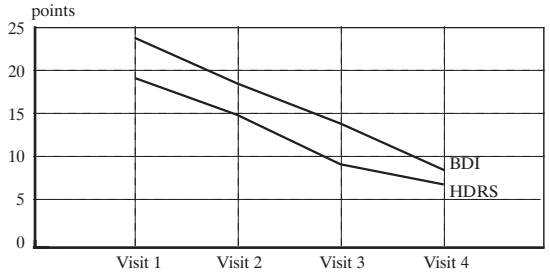


Table 1. **Intensity of fibromyalgia symptoms before and after therapy with mirtazapine in patients with fibromyalgia (n=26)**

Symptoms VAS	n=26		
	I	II	p<
Pain	7.92	5.0	0.0005
Morning stiff. (min)	67.9	48.7	0.0005
Fatigue	8.4	5.7	0.0005
Sleep disturbances	8.2	2.7	0.0001
Cold extremities	7.3	4.7	0.0005
Dryness of mouth	6.0	4.9	0.005
Perfuse sweating	6.4	4.2	0.001
Dizziness	5.6	2.7	0.0005
Headache	6.5	3.3	0.0001
Gastric problems	5.5	2.7	0.0005
Arrhythmia	7.4	4.3	0.0005
Irregular breathing	4.9	3.5	0.005
Paresthesia	8.0	4.2	0.0005
Urinary urgency	5.4	2.9	0.001

I – before enrolling into the study;
II–after 6 weeks treatment with mirtazapine

Table 2. **Correlation between reduction of depression (HDRS and BDI scales) and reduction of main fibromyalgia symptoms after 6 weeks of mirtazapine treatment**

	Reduction in HDRS	Reduction in BDI
Pain	0.72**	0.63**
Sleep disturbances	0.46*	0.42*
Fatigue	0.77**	0.69**
Morning stiffness	0.64**	0.53*

* p<0.05; ** p<0.005

The improvement expressed as ≥40% reduced intensity on all mentioned above parameters was observed in 10 patients (38%).

A significant amelioration after mirtazapine treatment was also noted in such somatic symptoms as cold extremities, dryness of mouth, perfuse sweating, dizziness, headache, gastrointestinal symptoms, arrhythmia, irregular breathing, paresthesia and urinary urgency what was shown in *Tab. 1*.

The mean weekly dosage of paracetamol in whole group of patients was reduced from 4.5 g taken during the week before treatment to 1.5 g during the last week (p<0.0005).

Eight patients complained of sleepiness and hypotension in the first days of therapy. In 5 cases the dose of mirtazapine was reduced to 15 mg daily and treatment was continued. Three female patients dropped out from the study after second visit on account of this side effect. Other side effects were not observed during mirtazapine treatment.

In the whole group of patients before treatment, there was a significant correlation between the intensity of depression, measured both with HDRS and BDI, and the intensity of pain and morning stiffness but not with the intensity of fatigue and sleep disturbances. The magnitude of reduction in depression after 6 weeks of mirtazapine treatment significantly correlated with the magnitude of reduction on all four main symptoms of FS, what was shown in *Tab. 2*.

Among 26 patients who completed the study, 18 of them (group A) had at least a moderately severe level of depression before mirtazapine treatment (HDRS score ≥18, mean 22.7) and 8 patients (group B) presented mild depressive symptoms (HDRS score <18, mean 13.1). *Tab. 3* shows the results of pain intensity, morning stiffness, fatigue and all other fibromyalgia symptoms before and after 6 weeks of treatment with mirtazapine in both groups of patients. Before treatment in patients from group A more intensity of fibromyalgia symptoms was observed in comparison with group B (range 7 to 47%) but the differences were not significant. Improvement after the therapy was statistically significant in both groups although in group A the mirtazapine seemed to be more effective (*Tab. 3*).

Discussion

The results obtained suggest that mirtazapine, 15-30 mg daily, is effective in reducing pain intensity, sleep disturbances, fatigue, intensity of vegetative and functional symptoms as well as the severity of depression in the majority of fibromyalgia patients.

The most robust effect of mirtazapine was observed on sleep quality. The sleep disturbances in FS are well known phenomenon, resembling those of depressive patients [34,35]. The beneficial effect of mirtazapine may be attributed to the action of this drug on 5-HT2 receptors. The analysis of 4, 5 and 6 HDRS items indicate, that treatment with mirtazapine leads

Table 3. **Intensity of fibromyalgia symptoms before and after therapy with mirtazapine (Group A: patients with ≥18 pts in HDRS; group B: patients with <18 pts in HDRS)**

Symptoms VAS	Group A n=18			Group B n=8			Difference IA and IB (%)
	I	II	p<	I	II	p<	
Pain	8.3	5.6	0.005	7.0	3.9	0.05	16
Morning stiff. (min)	79.2	61.2	0.005	42.5	23.7	0.05	47
Fatigue	8.4	6.1	0.005	8.4	5.0	0.05	0
Sleep disturbances	8.1	3.4	0.001	8.4	1.2	0.01	-4
Cold extremities	7.5	4.9	0.005	6.7	4.2	0.05	11
Dryness of mouth	6.5	5.6	0.05	4.9	3.5	0.05	25
Perfuse sweating	7.2	5.0	0.005	4.5	2.7	NS	38
Dizziness	5.9	3.2	0.005	4.9	1.7	0.05	18
Headache	6.9	3.6	0.005	5.6	2.6	0.05	19
Gastric problems	6.2	3.1	0.005	4.0	2.0	0.05	36
Arrhythmia	7.6	3.8	0.005	7.1	5.4	0.05	7
Irregular breathing	5.2	3.6	0.01	4.2	3.2	NS	20
Paresthesia	8.0	4.4	0.01	7.9	3.9	0.05	2
Urinary urgency	5.5	2.8	0.005	5.2	3.0	0.05	6

I – before enrolling into the study
II – after 6 weeks treatment with mirtazapine
NS – not significant

to improvement of sleep quality, especially problems with too early awakening.

We observed relatively high prevalence of depression symptoms among examined patients, 18 of them (69%) represented at least moderately severe level of depression (score ≥18). Depression was the second phenomenon in fibromyalgia patients, after sleep, which was influenced most favorably during mirtazapine treatment.

Important observation of our study is also significant improvement in other vegetative and functional symptoms, like pain, fatigue, headache, gastrointestinal complaints, paresthesia and urinary urgency. These disturbances are the reason of many complaints, significantly restricting life activity of patients. Another advantage of mirtazapine treatment is a possibility of significantly reducing the use of analgetic agents. Apart from hypotension in the initial period of therapy, there were no serious adverse effects during the treatment with mirtazapine in our study.

The issue of a possible relationship between therapeutic effect of mirtazapine in FS and antidepressant action of the drug can be answered positively. The magnitude of reduction in depression after 6 weeks of mirtazapine treatment in whole group of patients significantly correlated with the magnitude of reduction on all four main symptoms of FS. This would suggest a possibility of common pathophysiology of depression and symptoms of fibromyalgia.

In conclusion, our results indicate that mirtazapine can be an effective and promising method in the treatment of FS patients. These observations should be confirmed on the greater group of patients and in the double-blind placebo-controlled trial.

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Is a hypothermic effect of LY300164, valproate and phenobarbital evident in mice?

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Abstract

Purpose: **The aim of this study was to evaluate the influence of LY300164 (an AMPA/kainate receptor antagonist) administered alone or in combination with valproate (VPA) or phenobarbital (PB) on body temperature in mice.**

Material and methods: **The temperature measurements were performed in Albino Swiss mice injected with the respective drugs by using a rectal thermistor thermometer.**

Results: **LY300164, at the dose of 2 mg/kg, did not affect the body temperature of the examined animals. However, the combination of LY300164 (2 mg/kg) with VPA (165 mg/kg) resulted in a significant decrease in body temperature within 60-180 min after their peak of maximum anticonvulsant activity. Moreover, VPA (269 mg/kg) administered alone, evidently produced hypothermic effects at the times between 120-180 min after the peak of the maximum antiseizure effect. In contrast, phenobarbital administered alone or in combination with LY300164 did not affect the body temperature in the mice.**

Conclusions: **Hypothermia induced by LY 300164 combined with VPA may be useful in various central nervous system disease treatments.**

Key words: temperature monitoring, hypothermia, LY300164, valproate, phenobarbital.

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Introduction

The excessive activity of excitatory amino acid (EAA) neurotransmitters in the brain is thought to be an important factor involving in several central nervous system diseases such as: cerebral ischemia/hypoxia, trauma injuries, epilepsy, Huntington's chorea, Parkinson's disease and Alzheimer's disease [1,2]. There are numerous brain pathologies resulting from the imbalance between excitatory and inhibitory neurotransmitter system functioning in the brain [1,3]. For thirty years, an important role of EAA in epileptogenesis and seizure initiation, amplification or propagation has been documented [4]. Moreover, the neuroprotective effect of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5,7--methylisoxazole-4-propionic acid (AMPA)/kainate receptor antagonists have been demonstrated in several experimental models of epilepsy [5,6]. At present, the AMPA/kainate receptor antagonists seem to be more advantageous than NMDA receptor antagonists as to the acute neurotoxic effects produced by these agents [7].

In experimental and histological studies, it has been found that hypothermia protects the neurons against cerebral ischemia and traumatic brain injury, whereas conversely, hyperthermia exacerbates such brain pathologies [8]. The investigations, after the intracerebroventricular (i.c.v.) administration of NMDA, have revealed that this agent is responsible for the increase in brain temperature of examined rats [9]. This increment was inhibited by NMDA receptor antagonists, such as: MK-801 (dizocilpine) and (±)-2-amino-5-phosphonopentanoic acid [10]. In contrast, AMPA and kainic acid produced a biphasic effects on body temperature of experimental animals: short-lasting hypothermia followed by hyperthermia [11]. So, the suppression of hyperthermia by appropriate drugs might be useful during the treatment of patients with some brain pathologies in which the EAA are involved.

LY300164 {7-acethyl-3-(4-aminophenyl)-8,9-dihydro-8-methyl-7H-1,3-dioxazolo-[4,5-h][2,3]-benzodiazepine}; (Talampanel®), a non-selective antagonist of AMPA/kainate

receptors, enhanced the anticonvulsant activity of conventional and some novel antiepileptic drugs (AEDs) against maximal electroshock (MES) [12], aminophylline-induced seizures in mice [13] or amygdala-kindling rats [14]. Also, the substance was resistant to the convulsive action of sub-effective doses of aminophylline and strychnine when compared to conventional or novel AEDs (i.e. phenobarbital, valproate, diphenylhydantoin [15] or lamotrigine [16]). Therefore, in the present study we examined the effect of LY 300164 alone or in combination with phenobarbital or valproate on body temperature following the administration of this AMPA/kainate receptor antagonist in mice. The doses of the investigated AEDs and LY 300164 as well as the scheduled times, in which the experiment was performed, were previously determinate by Czuczwar et al. [17].

Material and methods

General

The experiments were conducted on female Swiss mice weighing 20-25 g. The animals were housed in colony cages with food (chow pellets) and tap water ad libitum. The laboratory temperature was 21 ± 1°C and the mice were kept on a natural light-dark cycle. The experimental groups consisting of 10-12 animals were randomly assigned. The procedure of temperature monitoring was carried out between 10:00 a.m. and 2:00 p.m. All experiments were approved by a Local Ethics Committee at the Medical University of Lublin.

Drugs

The AEDs used were: valproate magnesium (ICN Polfa Rzeszów, Poland) and phenobarbital sodium (Polfa, Warsaw, Poland). Valproate and phenobarbital were dissolved in sterile saline. LY 300164 ({7-acethyl-3-(4-aminophenyl)-8,9-dihydro--8-methyl-7H-1,3-dioxolo-[4,5][2,3]-benzodiazepine}; kindly supplied by Eli-Lilly, Indianapolis, IN, USA) was dissolved in sterile saline. All drugs were injected intraperitoneally (i.p.), in a volume of 10 ml/kg; valproate magnesium – 30 min, phenobarbital – 60 min, and LY 300164 – 15 min before the tests.

Measurement of animals' temperature

Before the test examination, the animals were pretrained three times a week to eliminate any handling-evoked variability in their body temperature. During this procedure, all mice received an i.p.-injection of saline. The temperature measurements were performed at a constant room temperature of 21 ± 1°C, and were performed in rectum of the animals with a thermistor thermometer (Elab, Copenhagen, Denmark); the probe was inserted into a depth of 10 mm, and maintained till a stabilization of temperature was achieved. The reference temperature was a mean temperature of three preliminary measurements taken consecutively at 10 min intervals. After the third measure, valproate magnesium and phenobarbital sodium or LY 300164 were administered to animals and the temperature was recorded. The control animals received always the respective amount of saline. Next, the temperature was monitored at 15, 30, 45, 60, 90, 120 and 180 min, after the first recording. Alterations in body temperature were presented as means ± SEM of at least 10 determinations for each time of measurement.

Statistics

Statistical evaluation of data was performed with repeated measures two-way analysis of variance (ANOVA) followed by Bonferroni a posteriori test.

Results

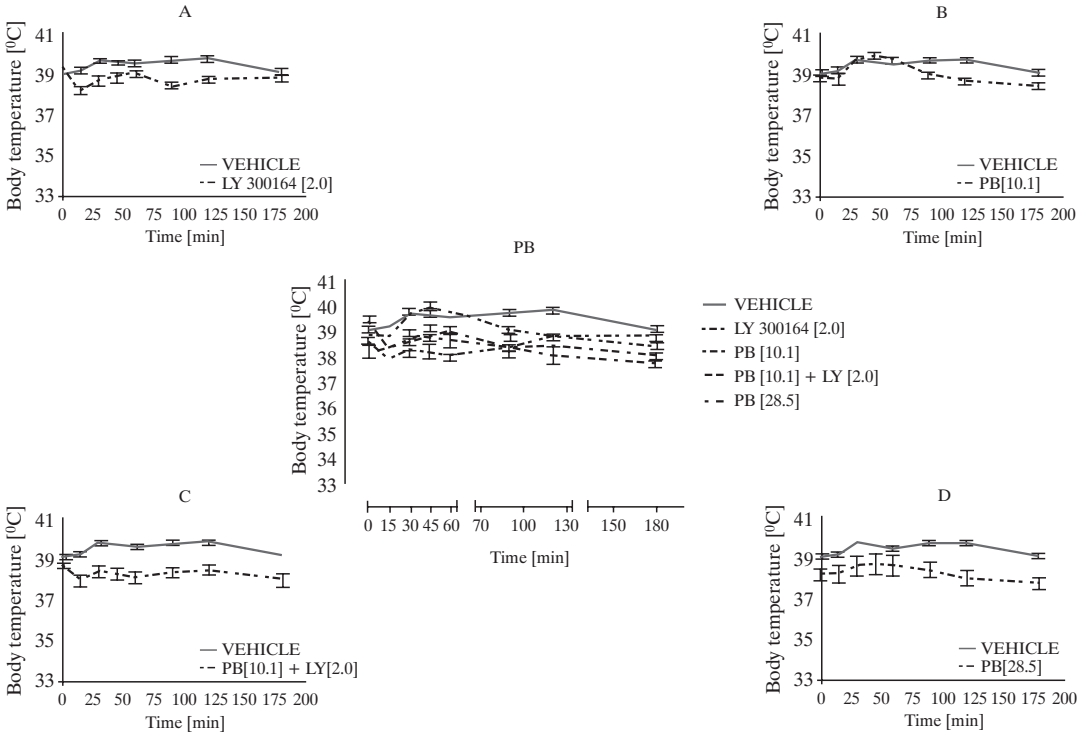
LY 300164 administered singly at the dose of 2 mg/kg did not affect body temperature of animals (*Fig. 1*; *Fig. 2*). Likewise, phenobarbital injected alone (28.5 mg/kg) did not significantly alter the body temperature. The combination of phenobarbital (10.1 mg/kg) with LY 300164 (2 mg/kg) did not influence this parameter in examined animals (*Fig. 1*).

Mean temperatures for LY 300164 and VPA administered alone or in combinations were calculated from 10-12 individual animals per group subjected to the temperature recording in various times after their peak of maximum anticonvulsant effects. The mean body temperature for the combination of VPA (165 mg/kg) and LY 300164 (2 mg/kg) was drastically lowered at the times of 90, 120 and 180 min. after their peak of maximum anticonvulsant activity. The difference between the mean temperature of control animals and those injected with the drug mixture (VPA + LY) achieved 4°C, 4.7°C, and 4.7 °C for 90, 120 and 180 min, respectively (at $P < 0.001$; *Tab. 2*). Simultaneously, the drug mixture decreased body temperature of the animals when compared with LY 300164 and VPA (*Tab. 2*). All remaining comparisons of the body temperature in the mice did not reach statistical significance for both, time-effect and drug-effect relationships with the repeated measures two-way ANOVA (*Tab. 2*; *Fig. 2*).

Discussion

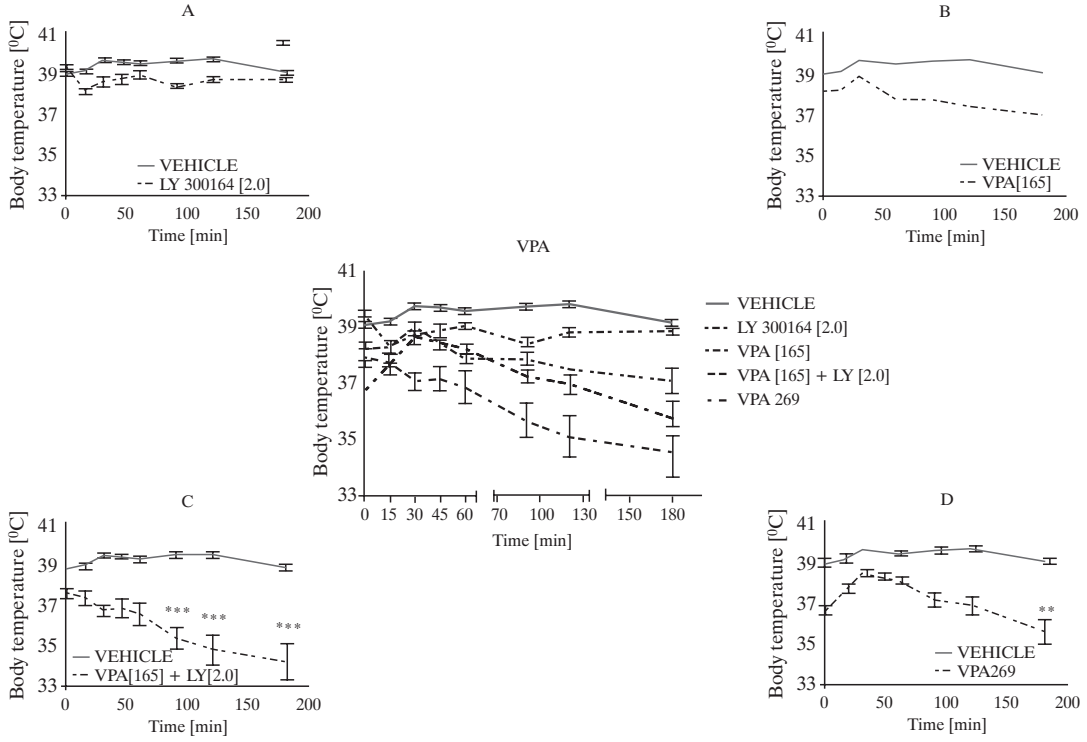
Our findings indicate that LY 300164 co-administered with valproate (at the dose of 165 mg/kg; providing a 50% protection against MES-induced seizures) produced a marked hypothermic effect. Moreover, valproate (269 mg/kg) injected alone, also reduced the body temperature in experimental animals. However, the mixture of valproate with LY 300164 showed longer and more expressed effects than valproate alone. In contrast, no temperature changes between animals administered with phenobarbital alone or in combination with LY 300164 were observed in our study. Since the results in our study were analyzed using two-way repeated measures ANOVA, both, time-dependent and drug-dependent relationships were estimated. Following this analysis, PB and LY 300164 alone or in combination did not significantly alter the body temperature of the animals as compared with control group. We are fully aware of the fact that the obtained results, despite the temperature difference ranging between 1-3°C among the analyzed groups, did not achieve statistical significance which was a result of the inter-group (individual) variance in body temperature after AEDs administration.

Figure 1. **Influence of LY 300164 and phenobarbital on body temperature in mice**



The temperature was monitored by rectal probe several times within the experimental period as follows: 0, 15, 30, 45, 60, 90, 120 and 180 min after the time of peak AED effect. The mice received LY 300164 (2.0 mg/kg) (A), or a combination of LY 300164 with PB (10.1 mg/kg) (C), had not significantly reduced the body temperature. Moreover, the temperature of animals subjected to the i.p. injection of PB (28.5 mg/kg) and (10.1 mg/kg) alone (B,D) did not differ from that of the control (vehicle-treated) mice. PB-phenobarbital

Figure 2. **Influence of LY 300164 and valproate on body temperature in mice**



The temperature was monitored by rectal probe several times within the experimental period as follows: 0, 15, 30, 45, 60, 90, 120 and 180 min after the time of peak AED effect. VPA at the dose of 269 mg/kg produced hypothermic effects at the times ranging between 120-180 min at $^{**}P<0.01$ (D). Moreover, the combined treatment of LY 300164 (2 mg/kg) with VPA (165 mg/kg) resulted in a significant decrease in animals' temperature at the times ranging between 90-180 min at $^{***}P<0.001$ (C). (For more details see also the *Tab. 2*). The body temperature of the mice injected with VPA (165 mg/kg) did not significantly differ from that of the vehicle-treated animals (A; B). VPA-valproate

***Table 1*. Effects of LY 300164, phenobarbital and its combination on body temperature in mice**

Time (min.)	Temperature (°C)			
	Control	LY [2.0]	PB [10.1]	PB[10.1]+LY[2.0]
0	39.1 ± 0.15	39.5 ± 0.10	38.8 ± 0.14	38.6 ± 0.10
15	39.2 ± 0.11	38.2 ± 0.16	38.8 ± 0.26	37.9 ± 0.26
30	39.8 ± 0.05	38.7 ± 0.25	39.8 ± 0.15	38.4 ± 0.35
45	39.7 ± 0.07	38.9 ± 0.23	39.9 ± 0.14	38.2 ± 0.31
60	39.6 ± 0.11	39.1 ± 0.17	39.8 ± 0.08	38.1 ± 0.22
90	39.7 ± 0.11	38.5 ± 0.16	39.0 ± 0.13	38.4 ± 0.23
120	39.8 ± 0.09	38.8 ± 0.16	38.8 ± 0.10	38.5 ± 0.30
180	39.1 ± 0.14	38.9 ± 0.13	38.5 ± 0.15	37.9 ± 0.30

Table data are presented as mean temperatures ± SEM of at least 10 determinations.
Statistical analysis was performed using two-way repeated measurements ANOVA followed by Bonferroni post-hoc test. Control – vehicle-treated animals; LY – LY 300164; PB – phenobarbital.

***Table 2*. Effects of LY300164, valproate and its combination on body temperature in mice**

Time (min)	Temperature (°C)			
	Control	LY [2.0]	VPA [165]	VPA[165]+LY[2.0]
0	39.1 ± 0.15	39.5 ± 0.10	38.2 ± 0.23	37.9 ± 0.28
15	39.2 ± 0.11	38.2 ± 0.16	38.3 ± 0.19	37.7 ± 0.37
30	39.8 ± 0.05	38.7 ± 0.25	38.9 ± 0.18	37.0 ± 0.32
45	39.7 ± 0.07	38.9 ± 0.23	38.4 ± 0.19	37.2 ± 0.44
60	39.6 ± 0.11	39.1 ± 0.17	37.8 ± 0.18	36.9 ± 0.58
90	39.7 ± 0.11	38.5 ± 0.16	37.8 ± 0.24	35.7 ± 0.59***
120	39.8 ± 0.09	38.8 ± 0.16	37.5 ± 0.18	35.1 ± 0.74***, a
180	39.1 ± 0.14	38.9 ± 0.13	37.1 ± 0.41	34.5 ± 0.93***, b, ‡

Table data are presented as mean temperatures ± SEM of at least 10 determinations. Statistical analysis was performed using two-way repeated measurements ANOVA followed by Bonferroni post-test. ***$P<0.001$ vs control group; [a]$P<0.01$ vs LY-treated animals; [b]$P<0.001$ vs LY-treated animals; [‡]$P<0.05$ vs VPA group. Control – vehicle-treated animals; LY – LY 300164; VPA – valproate

In experimental studies, several conventional AEDs have evoked also the hypothermic effects, especially if the drugs were administered at higher doses. For instance, a substantial reduction in body temperature has been noted following i.p. administration of diazepam (2-5 mg/kg), carbamazepine (20-50 mg/kg) and valproate (100-300 mg) [18]. On the contrary, lamotrigine, phenobarbital, diphenylhydantoin, felbamate, and gabapentin have not affected the temperature of experimental animals. Hence, our findings are generally consistent with those presented by Gareri et al. [18], confirming that valproate reduced the temperature, whereas phenobarbital had no impact on this parameter in experimental mice.

Since some NMDA and AMPA/kainate receptor antagonists have exerted strong hypothermic effects [19] and possessed the neuroprotective activity in both focal and global ischemic models [20], LY 300164 might be considered as a good candidate drug to share these two properties. Although, the agent per se did not produce the reduction in animal body temperature, but it considerably enhanced the hypothermic effect, when combined with VPA.

In conclusion, the reduction of body temperature by the mixture of LY 300164 with valproate may occur neuroprotective in both, cerebral ischemia and seizure models. Nevertheless, more advanced investigations are required to fully understand the real mechanisms to be responsible for the coexistence of seizures, neuronal loss and regulation of temperature in living organisms.

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Serum hyaluronic acid during lamivudine treatment in chronic hepatitis B

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Abstract

Purpose: We address the question whether lamivudine treatment modulates serum concentrations of hyaluronic acid and whether the pre-treatment HA level can give the information about the presumptive result of treatment and whether HA level evaluation can be useful in monitoring the antiviral therapy in chronic hepatitis B.

Material and methods: Forty-nine patients, 31 man, aged 40 ± 2.5 years were treated with 100 mg lamivudnie per day for 48 weeks. Serum hyaluronic acid level was determined using enzyme-linked binding protein commercial assay (Corgenix Inc., USA).

Results: The mean HA pre-treatment levels were higher than among controls (69.6 ± 11.6 ng/ml vs 36.5 ± 7.6 ng/ml, mean ± SEM) and correlated with AST activity, $p = 0.002$; GGT activity, $p = 0.006$; ALP activity $p < 0.001$; prothrombin time, $p = 0.01$; and peripheral blood platelets count, $p = 0.001$ but did not correlated with ALT activity. The pre-treatment HA concentration correlated also with interlobular necroinflammatory activity score, $p = 0.049$ and with fibrosis score, $p = 0.026$, according to Scheuer classification. The mean HA levels decreased gradually during lamivudine treatment, up to levels lover than among controls (26.3 ± 5.7 ng/ml). There were not significant differences in pre-treatment levels observed between patients neither with HBs seroconversion versus those without it, nor between patients with HBe seroconversion versus those without it and among patients with normalization of ALT activity versus ones without it.

Conclusions: Serum hyaluronic acid level decreases during lamivudine treatment both in patients with HBeAg seroconversion and without it; serum hyaluronic acid pre-therapy levels correlate with necroinflammatory lobular activity score and with liver fibrosis score; serum hyaluronic acid is of no predictive value for lamivudine therapy response; serum hyaluronan may be valuable complementary marker in chronically HBV infected patients.

Key words: hyaluronan, chronic hepatitis B, lamivudine.

Introduction

Effective treatment of chronic hepatitis B, despite the progress of recent years, remains one of the major world health problems [1].

Lamivudine, an oral nucleoside analogue, has an established role for treatment of patients with chronic hepatitis B [1-4]. Nevertheless the sustained response is achieved in limited number of patients and several factors, as low ALT activity, HBeAg presence, high viral load, liver necroinflammatory activity and liver HBcAg has been associated with low response to the treatment [1,5]. Even in cases of lack of the virological response the improvement in hepatic necroinflammatory activity can be seen after treatment [4]. Non-invasive markers of the liver fibrogenesis and fibrolysis could become clues to predict the subsequent clinical course of the patient, to estimate the likely and to determine the actual response to antifibrotic therapy [6]. Although liver biopsy is still regarded as the gold standard for the stage of liver fibrosis assessment it provides static information about fibrotic process and cannot be performed on a day-to-day basis [6]. Serum biochemical markers are ideal candidates for this purpose. The favored tests should be safe and easy to perform, to be done repeatedly and at short

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time intervals. Among many candidates evaluated hyaluronic acid (hyaluronan, HA) seems to be a promising one [6-9].

HA is a ubiquitous glycosaminoglycan of extra cellular matrix, originating from repeating disaccharide units ([D-glucuronic acid (1-β-3) N-acety-D-glucosamine (1-β-4)]n, [10,11].

The increase of serum hyaluronic acid concentration has been shown in various chronic liver diseases, suggesting that progressive liver damage can be identified early on and monitored by serum hyaluronan evaluation [12-14].

In our previous study we have demonstrated the positive correlation of HA level and the presence of liver steatosis [15], which occurence in chronic hepatitis C predispose to disease progression [16].

There were no studies on HA in chronic hepatitis B patients treated with lamivudine so far. In this paper we address the question whether lamivudine treatment modulates serum concentrations of hyaluronic acid and whether the pre-treatment HA level can give the information about the presumptive result of treatment and whether HA level evaluation can be useful in monitoring the antiviral therapy.

Table 1. **Baseline characteristics of the patients**

Age (years)	
Median	41
Range	18-70
Male sex n.(%)	31 (63)
Histological activity score	
Portal/periportal necroinflammatory activity	
Median	3
Range	0-4
Lobular necroinflammatory activity	
Median	2
Range	1-4
Fibrosis score	
Median	1
Range	0-3
Serum alanine aminotransferase (IU/l)	
Median	75
Range	7-350
Serum bilirubin (mg%)	
Median	0.93
Range	0.4-3.2
Serum albumin	
Median	3.92
Range	2.8-4.92

Material and methods

Patients

Patients with detectable hepatitis B surface antigen (HBsAg) and HBeAg in serum at the time of screening and for at least the previous 6 months, and with ALT activities that were 1.3 to 10 times the upper limit of normal for at least previous 6 months were eligible for the study.

The antigens HBs, HBe and antibodies HBc were detected by micro-enzyme immunological method (MEIA, ABBOTT, Germany).

Patients were excluded if they were younger than 18 years old, if they had hepatitis C or D or human immunodeficiency virus infection, if they had decompensated liver disease (defined by serum bilirubin level more than 2.5 times the upper limit of normal, a prothrombin time prolonged by more than 3 seconds and a serum albumin level lower then 3 g/dl or a history of ascites, variceal hemorrhage or hepatic encephalopathy); if they had evidence of autoimmune hepatitis or metabolic liver disease, if they received corticosteroids within 6 months before enrolment.

The study was carried out in accordance with the Helsinki Declaration and all patients enrolled gave informed consent. Local Ethical Committee approved the study.

Evaluation

Patients were evaluated at baseline, than at the beginning of treatment and at weeks 4, 12, 24, 48. Patients received 100 mg/day of lamivudine (Glaxo-SmithKline) for 48 weeks.

In 39 patients liver biopsy was preformed within 6 months before the enrolment.

Assays

The standard liver function tests, including total serum bilirubin, alanine aminotransferase (ALT) activity, aspartate aminotransferase (AST) activity, gamma-glutamyl transpeptidase (GGT) activity, alkaline phosphatase activity (ALP), serum total proteins and albumin, platelet count were measured using Cobas Mira instrument (Roche). Prothrombin time and prothrombin index (PI) was determined using Kselmed K–3002 (Poland). Clinical data of the patients are presented in *Tab. 1*.

The fasting serum hyaluronic acid concentration was assessed with an enzyme-linked binding protein assay (commercially available kit, Corgenix Inc.,USA) using a 'capture molecule' known as the hyaluronic acid binding protein, according to the producer.

Serum HA normal levels determined in the laboratory from 24 age and sex matched healthy subjects were 36.5 ng/ml ± 7.6 ng/ml (mean ± SEM).

The HBV-DNA was isolated from 200 μl of serum with commercial reagents for DNA isolating (GenElute Blood Genomic DNA Kit, Sigma). The HBV-DNA in serum was measured in the Clinical Molecular Biology Department of the Medical University of Białystok in Poland. The amplification products of conserve part of HBV genome were stained with brome ethydyne and than visualized and analyzed in the computer system UVI-KS400i/Image of PC (Syngen Biotech, USA).

Histologic score

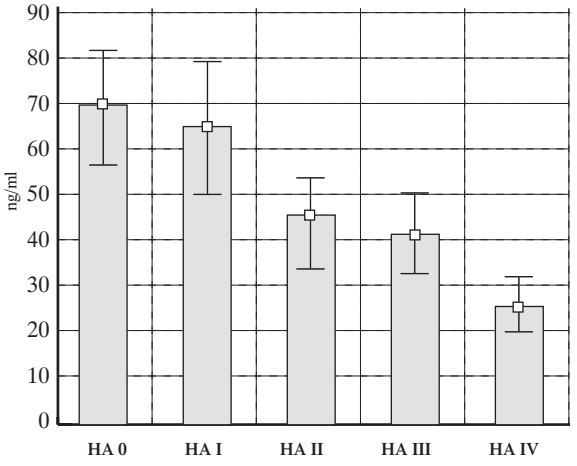
Liver biopsy was performed with the Menghini technique applying the 1,4 mm Hepafix needles (Braun, Germany). Biopsy specimens were fixed in 10% buffered formalin and embedded in paraffin. Serial sections (thickness 4-5 μm) were cut and stained with hematoxilin-eosin staining and picric acid staining for fibrosis evaluation.

Table 2. **Median and range of hyaluronic acid concentrations (ng/ml) in patients with chronic hepatitis B during 54 weeks lamivudine treatment; HA 0 – the level of hyalurinic acid assessed before treatment, HA I – after 4 weeks of lamivudine treatment, HA II – after 12 weeks, HA III – after 24 weeks, HA IV – after 48 weeks**

	Range (ng/ml)	Median (ng/ml)	p between HA 0 and HA I, HA II, HA III, HA IV
HA 0	1.0 – 326.5	37.5	
HA I	1.0 – 506.8	31.1	$p < 0.01$
HA II	1.0 – 319.8	20.1	$p < 0.001$
HA III	1.0 – 231.5	14.9	$p < 0.001$
HA IV	1.0 – 189.0	12.5*	$p < 0.001$

* p with norm

Figure 1. **The mean ± SD concentrations of hyaluronic acid (HA), in patients with chronic hepatitis B infection treated with lamivudine; HA 0 – the level of hyalurinic acid assessed before treatment, HA I – after 4 weeks of lamivudine treatment, HA II – after 12 weeks, HA III – after 24 weeks, HA IV – after 48 weeks**



The scoring system (0-4) of portal/periportal and lobular necroinflammatory activity and fibrosis was applied according to Scheuer [17].

Clinical endpoints

The endpoints included the loss of detectable levels of HBeAg and HBV DNA in serum and the appearance of antibody to HBeAg (referred to as HBeAg seroconversion) at the end of treatment; loss of detectable levels of HBsAg and appearance of antibody to HBsAg (referred to as HBsAg seroconversion) at the end of treatment; return of serum ALT levels to normal (≤40 IU/l).

Statistical analysis

Descriptive data are given as means ± SEM. The Mann-Whitney U test was used for statistical comparisons of quantitative variables as the distribution of hyaluronan values in our study group was not normal. The Pearson correlation coefficient and Spaermans's correlation coefficient were used to assess the degree of correlation of HA concentrations and other liver function tests and histology indexes, respectively. A two-tailed *p* value ≤0.05 was considered statistically significant.

Results

Forty-nine patients, 31 male and 18 female, aged 40.0 ± 2.5 years (mean ± SEM) accomplished study follow-up.

The mean serum HA pre-treatment level (69.6 ± 11.6 ng/ml) was higher than mean levels detected in healthy age matched controls (36.5 ng/ml ± 7.6 ng/ml; mean ± SEM).

The mean HA level 4 weeks after the treatment initiation decreased slightly up to 64.9 ± 14.5 ng/ml and than the mean HA levels decreased gradually during lamivudine treatment and the mean level detected at the end of the therapy was even lower than among controls (26.3 ± 5.7 ng/ml vs 36.5 ng/ml ± 7.6 ng/ml $p = 0.149$), *Tab. 2*, *Fig. 1*.

The pre-treatment HA levels correlated with AST activity, $p = 0.002$; GGT activity, $p = 0.006$, ALP activity, $p < 0.001$; prothrombin time, $p = 0.01$, and peripheral blood platelets, $p = 0.001$ but did not correlate with ALT activity, $p = 0.397$.

Virologic and biochemical response

Elimination of HBsAg was observed in two from 49 subjects (4.0%). The HA pre-treatment level did not differ significantly between those who eliminated HBsAg and those who did not.

The HBeAg seroconversion was detected in 17 from 49 individuals (34.6%). Similarly to the HBsAg seroconversion, the HA levels there were not statistically significant difference detected between those two groups of patients. The HA pre-treatment levels did not differ significantly between the responders to treatment and no responders.

The normalization of ALT activity was observed in 17 of 49 patients (34.6%). There were no differences in HA pre-treatment levels between subjects with ALT activity normalization and those with persistent elevated ALT activity, $p = 0.76$.

Histologic score and HA

The liver biopsy was performed in 39 patients. Serum pre-treatment level correlated with interlobular necroinflammatory activity score, $p = 0.049$ and with fibrosis score, $p = 0.026$, *Tab. 2*.

Discussion

The clinical course of HBV infection varies according to the phase of viral replication and the risk factors to which the patient is exposed, particularly superinfection with another hepatitis viruses [17]. Thus the histological diagnosis, not free from sampling errors, reflex only current status of the disease and every individual case should be carefully regularly monitored. So the non-invasive markers of prognostic value are needed. Hyaluronic acid fulfill those criteria, at least in some clinical conditions, e.g. among cirrhotic patients infected with HCV [14].

Table 3. **Correlation expressed through r-value and it's significance (* $p < 0.05$;** $p < 0.001$) between analysed biochemical indices and hyaluronic acid in patients with chronic hepatitis B before treatment**

	r-value in respect to HA (ng/ml)	
	mean ± SEM	r
Bilirubin (mg%)	1.1 ± 0.1	0.18
ALT (U/L)	119.0 ± 13.2	0.13
AST (U/L)	81.3 ± 9.1	0.42*
ALP (U/L)	103.8 ± 7.1	0.69**
GGT (U/L)	63.8 ± 10.3	0.4*
Prothrombin time	13.7 ± 0.2	0.37*
PI (%)	89.9 ± 1.5	-0.29
Total protein (g%)	7.1 ± 0.1	-0.15
Albumin (g%)	3.9 ± 0.1	-0.53*
Gamma-globulin (g%)	1.5 ± 0.1	0.54*
PLT ($x10^3/\mu L$)	161.8 ± 7.1	-0.5**
HGB (g/L)	14.6 ± 0.2	-0.07
Histological scores		
Lobular inflammation	2.72 ± 0.16	0.36*
Periportal inflammation	1.69 ± 0.13	0.30
Fibrosis	1.48 ± 0.13	0.40*

The histological activity index improved after one year lamivudine treatment in majority of patients regardless the HBeAg seroconversion [3,4]. Although in our study the end-point liver biopsy was not performed serum HA levels decreased significantly in all patients observed, both those who eliminated HBeAg and those who did not.

We have shown the positive correlation of serum HA concentration and lobular inflammation index and fibrosis index. The correlation of HA with fibrosis score was demonstrated in asymptomatic chronic HBV carriers [8] and in patients chronically infected with HBV and HCV viruses [18-20].

The rise of serum HA concentrations is believed to be caused both by increased synthesis and decreased removal by sinusoidal endothelial cells.

There is no available literature data on HA during lamivudine treatment. However HA levels during interferon treatment were in disagreement with ours where the increase of HA level was observed [21]. Although Zohren's et al. [21] follow-up included also patients with active cirrhosis among whom the increase was higher than among individuals with chronic persistent hepatitis B.

In our work we have demonstrated the positive correlation of serum concentrations HA and other biochemical parameters: AST, ALP, GGT, gammaglobulin, and negative correlation with serum albumin concentrations and platelets count. It is in line with observation among cirrhotic patients were the correlation of HA with albumin concentrations and platelets count was found but not with ALT activity [22].

There is several works on HA value as a prognostic marker in chronic hepatitis C patients treated with interferon [19,20]. Ueno et al. [19] hypothesized that serum HA levels, reflecting also hepatic sinusoidal capillarisation may explain the worse response to INF. The occurrence of hepatic capillarisation impair the movement of inflammatory cells and cytokines, including heterogeneous INF from the hepatic sinusoids to the space of Disse.

In our study the initial HA level was relatively low in comparison with chronic hepatitis C patients [19,20] 69.6 ± 11.6 (mean ± SEM) which may explain the lack of significant differences between responder and non-responders observed.

Although we did not demonstrate the prognostic HA value for lamivudine treatment response, HA seems to be a valuable complementary test in monitoring chronically HBV infected patients.

Conclusions

Serum hyaluronic acid level decreases during lamivudine treatment both in patients with and without HBeAg seroconversion.

Serum hyaluronic acid is of no predictive value for lamivudine therapy response.

Serum hyaluronan may be valuable complementary marker of liver fibrosis and necroinflammatory activity in chronically HBV infected patients.

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Falls amongst older people living in the community

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Abstract

Purpose: **Falls (instability) – as well as immobility, incontinence, intellectual impairment, depression and visual and auditory impairment are considered to be serious problems in the field of geriatrics. They have multiple causes, tend to reoccur, have no simple cure and make the older person dependent on others for care. This paper presents the results of the study on the prevalence of falls and their determinants in communities of older people (aged 75 and over).**

Material and methods: **The study design: cross-sectional questionnaire study and survey. The studied population lived in two chosen areas (urban and rural). The questionnaire, as well as instruments to verify the respondent's skills and functional ability, were used. Doctors and nurses employed in the studied areas were interviewers.**

Results: **457 randomly selected older people (228 from the rural area and 229 from the urban one) took part in the study. Falls in the course of the last year were reported by 45.1% of the group; more frequently by people living in the rural area (58.3% versus 31.9% in the urban one), by women (66.7% versus 44.8% in the men's group) and by people with different disabilities (cognitive impairment, locomotive disabilities, ADL-dependence, visual and auditory impairment), with poor self-reported health status and living arrangements. The score according to Tinetti's test--conducted in the rural areadetermined the frequency of the falls reported.**

Conclusions: **The study has confirmed that falling is an increasing hazard for older people, especially for women, for people living in rural areas and for those with different mental and physical disabilities.**

Key words: falls, community dwelling older people.

Introduction

Falls (instability) – as well as immobility, incontinence, intellectual impairment, depression, visual and auditory impairment are considered to be serious problems in the field of geriatrics. They have multiple causes, have chronic reoccurrences, no simple cure and make the elderly person dependent on others for care, and their quality of life deteriorates [1]. This also constitutes a large problem in the area of public health due to the frequency, consequences, and cost for essential medical attention. Falls are the main cause of death caused by accidents for those over 65, and the percentage of fatalities as a result of the accidents has risen significantly, regardless of the gender. In groups of people over the age of 75, these falls constitute 70% of the fatalities caused by accidents [2]. Fractures and bruises are the most common injuries resulting from falls; in groups of older people, there is often a need to be hospitalized as a consequence [3]. Over 90% of the femoral bone fractures are the results of such falls and they occur most often in the over 70 age group [4].

The aim of this paper is to present the results of a study on falls amongst the older population living in the community, rather than health care institutions, the frequency of the falls as well as the factors that influence them.

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Material and methods

This research constituted a part of the cross-sectional research devoted to the large geriatric problems in older persons. The populations studied lived in two chosen areas – urban (an urban district of Białystok) and rural (the municipality of Sokółka, not including the town of Sokółka), both with high percentages of demographic senility – those over the age of 65 and over made up 27.9% of the urban population and 18.5% of the rural population.

A representative sample of those living in the communities who were over the age of 75 was chosen: 299 people from the urban area and 313 from the rural area.

A questionnaire taking into account the following: 1) the social – demographic evaluation, 2) the occurrence of falls and other disabilities pertaining to older people, 3) the occurrence of chronic illnesses, 4) pharmacotherapy, and 5) the evaluation of the respondent's skills and functional ability with the help of a functional ability scale was used.

The following instruments were used to rate the respondent's functional ability:

- The EASY-Care questionnaire – used mostly to evaluate functional ability in the range of – ADL, Activities of Daily Living [5]. This took into account both instrumental activities ADL (I-ADL) – cleaning, meal preparation, shopping, using the telephone, taking medication, moving outside the home etc., as well as basic ADL (P-ADL) – being able to take care of oneself (eating, incontinence, using the toilet, bathtub/shower). In terms of ADL, the respondents were categorized as: able-bodied, dependent on others for care only in I-ADL (if there was an occurrence of at least one instrumental disability, but not in the self-care activities) or dependent on others for care in I-ADL and P-ADL (if there was an occurrence in at least one self-care activity). If sporadic urinary incontinence was the only disability, then the respondent was categorized as able-bodied.
- The scale of movement according to J. Piotrowski [6] – placed the respondents into 1 of 4 groups. Group I – persons able to move freely at home and outside the home; group II – persons moving freely around the home but having difficulties moving outside the home; group III – persons able to move 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 according to Katzman [7] – the test results were on the following scale: from 0 to 10 points showed a normal state or slight cognitive impairment, from 11 to 28 points – a moderate to serious cognitive impairment in the respondent.
- GDS - The Geriatric Depression Scale [8] – the emotional state of the respondent was rated in according to the following scale: from 0 to 5 points as a normal emotional state and a suspected state of depression with a rising tendency from 6 to15 points.
- Tests measuring the risk of falling according to Tinetti [9], used in the case of the urban respondents, as an element objectifying the functional impairment.

Doctors and nurses employed in the studied areas were interviewers.

The STATISTICA 5.0 [10] program was used to analyze the collected data. The Chi square Pearson test was used. A p-value of 0.05 or lower was considered to be statistically significant.

A detailed description of the research methodology was presented in an earlier publication [11].

Table 1. **Age and gender structure of examined groups in urban and rural areas**

	Urban [N=229]		**Rural** [N=228]	
	n	%	n	%
Age	p=0.02*			
75 - 79 years old	139	60.7	109	47.8
80 - 84 years old	44	19.2	67	29.4
85 - 89 years old	36	15.7	36	15.8
+ 90 years old	10	4.4	16	7.0
Average age [in years]	79.8±5.0		80.8±4.8	
Gender	NS*			
Male	82	35.8	87	38.2
Female	147	64.2	141	61.8

*Chi square Pearson test
NS – the differences are statistically insignificant

Results

463 persons took part in the research project focusing on serious problems in the field of geriatrics: 233 from the rural area (R) – 76.9% of the respondents, and 230 from the urban areas (U) – 74.4% of the respondents. Responses to the question of falls within the last twelve months were only given by 457 respondents (229 in the urban area and 228 in the rural area).

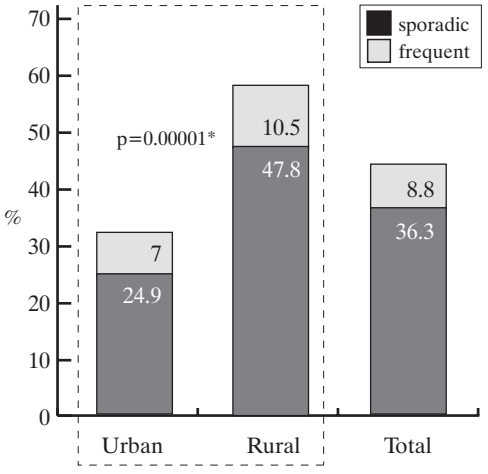
Age and gender structure of the examined groups are presented in *Tab. 1*. In both areas, most of the respondents were women (U – 64.2%; R – 61.8%), and in the case of the rural areas, there was a larger number of older sub-groups (52.2% persons 80 years of age and older versus 39.3% in the urban). The age structure in the male and female groups was similar in both of the studied areas. A detailed description of the social and demographic characteristics was presented in an earlier publication [12].

45.1% of the respondents claimed to have fallen and 8.8% of those examined stated that the falls were frequent (at least a few times during the year) – *Fig. 1*.

The frequency of falls (sporadic or frequent) was correlated with some of the socio-demographic parameters and is presented in *Tab. 2*. The evaluation of the respondent's state of health and their functional ability is presented in *Tab. 3*.

The frequency of falls increased in the older sub-groups (especially the falls rated as frequent), but not significantly. The gender of those interviewed did have an essential influence on the number of falls. 39.6% of the female respondents and 30.8% of the male respondents stated that the falls were sporadic, whereas 11.8% of the female and 3.5% respondents claimed that the falls were frequent. Significantly more often this prob-

Figure 1. **The prevalence of falls during the last 12 months in examined older people in rural and urban areas (in %)**



*Chi square Pearson test

Table 2. **Frequency of falls during the last 12 months and age, gender and living arrangements (N=457)**

Falls in the interview	None [%]	Sporadic [%]	Frequent [%]
Gender		p=0.0003*	
Male (n=169)	65.7	30.8	3.5
Female (n=288)	48.6	39.6	11.8
Age Group		NS	
75-79 years old (n=248)	58.5	351	6.4
80-84 years old (n=111)	53.2	35.1	11.7
85-89 years old (n=72)	50.0	41.7	8.3
+ 90 years old (n=26)	42.3	38.5	19.2
Place of residence		p=0.00001*	
Urban (n=229)	68.1	24.9	7.0
Rural (n=228)	41.7	47.8	10.5
Scale of living standards		p=0.006*	
Good (n=317)	60.2	31.9	7.9
Average (n=117)	45.3	43.6	11.1
Poor (n=23)	30.4	60.9	8.7

* Chi square Pearson test

Table 3. **Frequency of falls during the last 12 months and state of health / functional ability (N=457)**

Falls in the interview	None [%]	Sporadic [%]	Frequent [%]
Sight		p=0.00001*	
Fully sighted (n=284)	64.4	32.4	3.2
Partially sighted/blind (n=173)	39.3	42.8	17.9
Hearing		p=0.00001*	
Sound of hearing (n=266)	64.3	29.3	6.4
Hard of hearing/deaf (n=191)	41.9	46.1	12.0
Movement		p=0.00001*	
Group I (n=216)	74.5	25.0	0.5
Group II (n=168)	36.3	50.6	13.1
Group III (n=60)	45.0	35.0	20.0
Group IV (n=13)	15.4	46.1	38.4
Cognitive functions		p=0.00001*	
Normal state (n=361)	61.2	33.5	5.3
Suspected dementia (n=94)	30.9	46.8	22.3
Rating the state of health		p=0.0001*	
Good (n=88)	71.6	26.1	2.3
Average (n=235)	60.0	34.9	5.1
Poor (n=134)	35.1	45.5	19.4
ADL functional ability		p=0.00001*	
Able-bodied (n=107)	80.4	18.7	0.9
I-ADL dependent (n=108)	63.9	33.3	2.4
I-ADL and P-ADL dependent (n=242)	39.7	45.4	14.9
Points on the Tinetti scale (n=219 – only in the rural area)		p=0.0001*	
<19 points (n=145)	32.4	53.8	13.8
19-25 points (n=37)	59.5	37.8	2.7
>25 points (n=37)	67.6	32.4	0.0

* Chi square Pearson test

lem was reported by the respondents from the rural area then from the urban one (58.3% versus 31.9%). The unfavorable living conditions may also be a risk element in the number of falls (causes of the "outdoor" falls). Those who rated their living conditions as poorer had a significantly higher frequency of the falls.

As expected, the falls were substantially higher in those respondents who showed to be in worse mental and physical condition. This connection was also noted in both the case of functional cognitive impairments, as well as in cases of physical impairments (worse physical condition and mobility and difficulties in performing ADL tasks). Falls were also more noted amongst those respondents who rated their state of health as worse as well as an impairment of their hearing and sight skills.

As a survey objectifying the level of functional ability, the fall risk rate according to Tinetti was carried out on the group of 219-people from the rural area. The results gained from this test differentiated the respondents with regards to the frequency of noted falls during the interview were statistically significant.

The characteristics of the respondents with frequent falls is presented in *Tab. 4*. Women living in the rural areas with a low score on the Tineetti scale and persons dependent on others for care in self-care activities and rating their vision and general state of health as poor-constituted a large majority. In half of

***Table 4*. Characteristics of older persons reporting frequent falls during the last 12 months (N=40; 8,8% of the whole group studied)**

Characteristics	[%]
<19 points on the Tinetti* scale	95.2
I-ADL+P-ADL dependence	90.0
Female gender	85.0
Poor vision	77.5
Poor state of health	65.0
Residing in a rural area	60.0
Suspected dementia	52.5
III/IV movement group according to J. Piotrowski	42.5
Poor living conditions	37.5
Age - 85+	27.5

* N=21 (pertains only to the rural area)

the cases, cognitive dysfunctions and movement impairments were noted.

Discussion

The epidemiological studies conducted amongst the older persons living in the community show that at least 30-40% [4,13,14] of the respondents experience a fall at least once a year. Confirmed higher risk fall factors are: senility (advancement of age), the female gender, cognitive function impairments, as well as polypragmasy, and chronic ailments [15]. Due to the fact that most of these factors are chronic and inter-related, it is common for one person to experience more than one fall.

The frequency of noted falls in the interviews conducted with older persons were similar to other epidemiological studies [13,14,16]. A rise in the number of falls was observed as the age of the respondents increased but was not statistically significant. The study results confirmed, however, that there was a higher frequency amongst women. This is a major problem in women with fractures as a consequences of the intensified osteoporological changes.

Another important factor influencing the number of noted falls was the place of residence – there was a significantly higher number of falls noted in the older persons residing in the rural areas. Why was such a difference noted in the number of falls between the rural and urban areas? One of the possible reasons is perhaps a slightly more advanced age of the respondents interviewed in the rural areas and an objectively worse state of health and functional ability. Both the rural and urban areas differed significantly in the occurrence of the impairments. A substantially higher number of persons residing in the rural areas claimed to have sight and hearing impairments as well as being dependent on others for care in ADL tasks [17].

Two causes of falls have been accepted: the internal (organic) and the external (environmental) [18]. The second could be, for example, an untidy apartment (objects on the floor), rugs that move across the floor easily, a crooked or slippery floor, carpet skirting boards that protrude, stairs and poor lighting. These factors are responsible for over 50% of the falls noted amongst older persons [16]. One of the observed causes for the higher number of falls noted in those respondents living in the rural areas – apart from the health factors – may have been environmental causes (i.e., uneven flooring, poor lighting in the residence, uneven pavements, country roads). It is also worth noting that the respondents from the rural areas rated their living conditions as poorer than those from the urban areas (9.65% in comparison to 0.44% from the urban areas, $p<0.00001$).

A majority of the falls are caused by the overlapping of the internal and external factors, and their relative significance in a specific case is dependent on the age, state of health and functional ability of the person who fell. While the persons who experience falls within their residences are most often disabled (i.e. as shown in the prospective results – with a higher risk of death), those who experience falls outside of their residences are most often able-bodied persons and no increase in the risk of death was noted [19]. This duality in the fall factors in older persons can be confirmed by the conclusion stemming from this study that despite the fact of better movement skills and functional abilities of those interviewed in the rural region [17], where the falls noted were higher, as well as the fact that in those persons who experienced frequent falls – barely half had limited movement skills (groups III/IV according to J. Piotrowski).

The programs that are to help prevent falls in older persons should encompass screening of the persons at risk, and then an intervention aimed both at the internal and the external fall factors [20]. This type of intervention would decrease the risk of subsequent falls significantly as well as limit the negative effects of these events on the physical conditions of older persons, if they should arise [21]. An adequate pharmacotherapy needs to be coordinated [22], an instructional course on how to prevent falls needs to be conducted, the elimination of all of the environmental factors that can be eliminated should be carried out, and a program of appropriate exercises needs to be implemented [23,24]. The significance of the full geriatric evaluation in older patients hospitalized as a result of a femoral fracture as well as a subsequent intervention aimed towards eliminating the risk factors should be underlined [25]. These tests have shown that a useful tool helping to identify persons with a greater fall factor can be the fall risk factor test according to Tinetti.

Conclusions

1. The study confirmed the large prevalence of falls in older persons living in the community, outside of health care institutions.
2. The factors connected with a higher frequency of the falls in the examined groups were: living in rural areas, the female gender, and a worse mental and physical state of the respondents.
3. The fall risk factor test according to Tinetti constitutes a helpful tool in identifying of persons with a greater fall risk.

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Vascularization of the penis of a man

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Abstract

Purpose: **The study of the features of the blood supply of a penis of the man.**

Material and methods: **Macromicropreparation, angiography, corrosion method, morphometry, statistical method.**

Results: **The penis has three venous collector-executing outflow of blood. First of them is submitted surface dorsal vein, which is shaped from small-sized venous vessels of skin, subcutaneous fat and surface fascia of penis. The beginning deep dorsal vein, which will derivate second venous collector, gives veniplex of head of the penis. The spongy veins outstanding as third venous collector, reach the bulb of penis, where they receive small-sized bulbar vein. The arterial blood supply of penis happens at the expense of external and internal pudendal arteries. The external pudendal artery starts from an internal wall of femoral artery on 2.5-2.7 cm below inguinal ligament. In some cases (8%) the artery starts by two trunks – forward and back. The internal pudendal artery is main source of blood supply of penis of the man. It removes from forward trunk of internal iliac artery independently in 50% of cases. In remaining cases it or removes from this artery by one trunk with lower gluteal (36%), common trunk with the upper and lower gluteal arteries (4%), or with upper gluteal (8%), or with obturator artery (2%). Besides in the arterial blood supply of penis take part bulbar, urethral, dorsal and deep arteries of penis.**

Conclusions: **The penis receives blood from external and internal pudendal arteries, which are very variable. The venous blood of the penis flows off in three types of veins.**

Key words: penis, veins of penis, arteries of penis, erectile dysfunction.

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Introduction

The development of the medical technology has deepened the knowledge of organic violations of gears of erection. It was straightened out, that more than 50% from them cause vascular disorders [1-4]. It has given a particular push to more detailed learning extra- and intraorgans vessels of the penis. At the same time, the problems of vascularization and relationships of blood vessels of the penis have been investigated not enough, and in number of cases the results of the researches are contradictory [5-8].

The purpose of the research is to study of the arteries and veins of the penis of a man.

Material and methods

As the materials for the research penises from 160 corpses of men aged from 17 to 74 that had died unexpectedly were used. Methods of the research are:

1. Cavernozometry

According to Wespes E [9], infusion cavernozometry, which use in clinic, maybe exploit on the corpse not later than 24 hours from the moment of death. It is accepted to consider 150 - 200 ml/min as conditional norm of infusion cavernozometry in this case, more than 200 ml/min and absence of artificial erection as pathology of venous outflow. Therefore, with the purpose of building the artificial erection each corpse was

exposed cavernozometry for definition of functioning of venous outflow from the penis. The nature of the method consists in the following. One of cavernous bodies in range of prepuce is punctured by a needle with the diameter of foramen of 0.8 mm, which with the help of polychlorvynil tube paired to the device of artificial circulation and pot with solution. Perfusion within 3-5 minutes was carried out by warm (37°C) normal saline solution in the following parameters: 1) 150 ml/min; 2) 200 ml/min; 3) more than 200 ml/min. The first and second parameters were accepted for conditional norm, and more than 200 ml/min or absence of artificial erection spoke about infringement of venous hemodynamics of penis.

2. Macromicropreparation

With the purpose of specification of some topographical features and simplification of the subsequent preparing of the fine branches the filling of the veins of the penis (after preliminary erasion from the organ of the residual blood with the help of the lavage by the normal saline solution) with the solution of leaden bleached on monomer "Etakril" in the ratio 1:5 with addition 2 parts of polymer was carried out. Injection mass was introduced backward through the deep dorsal vein of the penis by access from the perineum under pressure of 50 mmHg. Then penis was exposed to level-by-level preparing under binocular supraforehead magnifier.

The received data were recorded in the protocols, where the some variants of the arteries and veins of the penis were described; their sketches and photographing were carried out.

3. Roentgen angiography

For reception of roentgenograms of vessels of the penis was used roentgen contrast mass of the following structure: a) leaden suric 30%, zinc bleached 15%, rest turpentine; b) leaden bleached 40%, rest turpentine. The injection of veins of the penis was carried out as follows. In a position of the corpse on a back with bent in patellar and hip joints and divorced femurs, access to deep dorsal vein of the penis carries out by the semicircular cut with the establishment at ischial tuber and apex at root of the scrotum. The deep dorsal vein of the penis excretes in the triangle formed by superficial transversal muscle of the perineum, the bulbo-spongy and ischial-cavernous muscles. This vein, having removed by Farabef's hook the spongiform body of the penis, take on the forceps and the forward wall dissect transversally by scissors. Then through the cut introduce the subclavial catheter of the diameter of the foramen 0.3 sm and advance it against the stop with venous valve, which, as a rule, settles down at the level of the basis of the organ. Through the catheter the metal explorer of the diameter 0.2 cm advance and blast the valve. Then remove explorer and the special device attach to the catheter, allowing to supervise pressure of the introduced contrast agent, which freely fills in all veins of the penis, due to presence of anastomoses between superficial both deep dorsal, and spongiform veins. Thus complete architectonic of the veins of the penis turns out without contrasting of the cavernous bodies, dui to the perforate veins are submitted as the turned funnel, that interferes to hit of contrast in the cavernous bodies.

4. Morphometry

After anatomical preparation and the performances of roentgenography with the help of MBS-2 measured the diameter of dorsal veins (at the basis of the organ), circumflex ones (at the place of run them in deep dorsal vein), spongiform veins (at the level of the bulbus of the penis), veins of the retroglandular plexus (at the level of their coalescence). The detailed study of topographo-anatomical features of the internal pudendal artery and its branches was carried out: their position both mutual relation with veins and nerves, length and diameter on different departments, depth of the lying, definition of the projection of the internal pudendal artery in relation to anatomical orienteer of the perineum. In each age-grade the vessels were exposed to the morphometry: length and diameter, as the basic parameters capacitor and bandwidth of veins; depth of the wall and shells, amounting it.

5. Histological method

For study of inner structures of the vascular canals of the penis the tissue specimens on the standard procedure were produced. For this purpose at the level of the head, cervix, middle third of body and the basis of the organ were cut out slices by depth of 10 mm. The received fields of the penis were fixed in 10% solution of neutral Formalin. After flush in water and the deaquations in alcohol of growing concentration were filled in paraffin. Sections by depth of 5 micron from paraffin trochleas prepared on the microtome. The microstructure of the wall of vessels studied with use of histological procedures: coloring of paraffin sections by hematoxylin – eosin, by van Gizon.

6. Statistical method

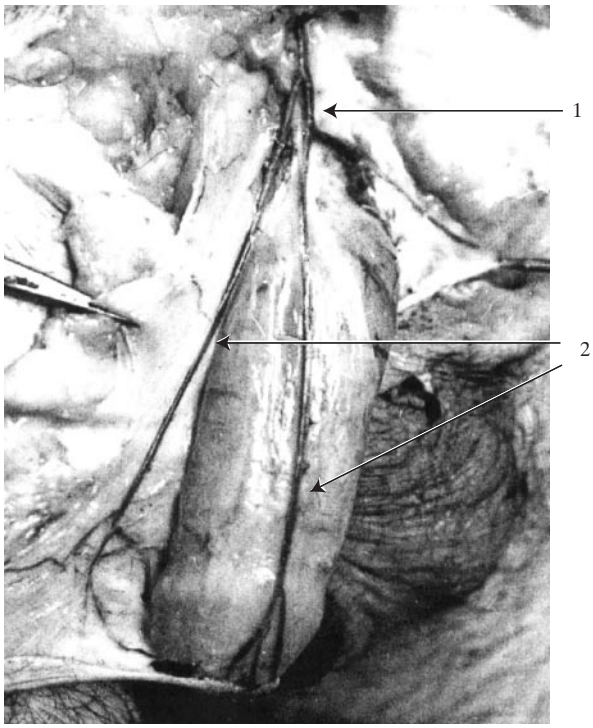
All data are subjected to statistical processing. Thus average arithmetic size, average square deflection, mean error average arithmetic, and coefficient of variation were calculated. The specified statistical parameters are received on the computer with the help of the program "Statistica 5.5". Reliability of difference between separate bunches determined with the help of criterion T. Under the Student's tables the probability (P) of reliability of differences of compared sizes estimated. For the minimal probability of differences was accepted $p < 0.05$.

All anatomical terms are given under the International nomenclature.

Results

The analysis of the received results gives the basis to state the number of situations, not enough illustrated and explained in the literature. A penis has three venous collector-executing outflow of blood. The first of them is submitted by superficial dorsal vein, which is formed from small-sized venous vessels of skin, subcutaneous fat and superficial fascia of penis. One (36.5%) or two (63.5%) trunks can submit it. If the superficial dorsal vein is submitted by one trunk, it is located on the dorsum penis, if it consists of two trunks, then they lie on upper-lateral area of the organ. Placing between superficial and deep fascias of the organ, the superficial dorsal vein irrespective of the quantity of trunks, reaches for the basis of the penis, then turns

Figure 1. **Veins of the penis of man: 1 – subcutaneous veins of anterior abdominal wall; 2 – superficial dorsal veins**



externally, and in the field of hypodermic slot of the femur runs accordingly into the left or dextral large hypodermic vein of the femur. At presence of one trunk, the superficial dorsal vein is a more often (82.9%) run into the left large hypodermic vein of the femur. In 16.9% of cases the superficial dorsal vein is poured in hypodermic veins of the abdominal wall (*Fig. 1*).

The beginning deep dorsal vein, which is the derivate of the second venous collector, gives the head of penis veniplex. Divided by connective tissues trabecules, veins of the head of the penis, which on shears imitate cells of cavernous tissue, sequentially merge from small-sized in larger and leave from under crown on the back of the organ. These trunks also shape the head of penis veniplex, which lies between deep fascia of the penis and white of cavernous bodies. The quantity of the venous trunks can vary from two up to seven. The average diameter of the veins of this veniplex is 1.87 ± 0.01 mm. These venous trunks merge among themselves and form the deep dorsal vein (*Fig. 2*). It represents rather large vessel with the diameter of 4-5 mm with thick walls, which are densely fixed to white of cavernous bodies. Not far from the basis the deep dorsal vein has the valve opening proximally, and for the basis of the doors of the valve the thickening of the walls of the vein is scored at the expense of the growth of the muscular coat. The deep dorsal vein in some cases (28.2%) can be divided into two trunks with the average diameter of 2.69 ± 0.14 mm, from them at a level of mean third of penis (81.05%) or for the basis (18.95%). According to our data, in the deep dorsal vein, in it distal and middle third, the circumflex veins, which carry blood from cavernous bodies, run. There is an anastomosis between the superficial dorsal and the deep dorsal veins in the area of the prepuce in 87.6% of cases. (*Fig. 3*). It was shown, that the circumflex veins with the average diameter of 1.65 ± 0.06 mm in the majority cases (96.2%) are shaped of junction of two trunks: the perforation vein of the cavernous body and the vein leaving the spongy body in area of the urethral sulcus. In 3.8% of cases the circumflex veins are direct prolongation by one only perforation vein passing through a dense white. As the result of the carried out research, we detect in 19.86% of cases the circumflex vein with the average diameter of 1.75 ± 0.01 mm, which was shaped in the field of mean third of cavernous body, placing on lateral area of penis, and run in deep dorsal vein at the basis of the organ, i.e. behind of venous valve. On our material in the majority cases (80,14%) the circumflex veins run into the deep dorsal vein in its distal and middle third in numbers from four up to eight pairs. Besides it is necessary to note, that at the left circumflex veins runs a little bit more, than on the right ($p > 0.01$). Having accepted in itself circumflex veins the deep dorsal vein reaches a place of divergence of cavernous bodies on two pinches and further passes in interval between arcuate ligament of pubis and transversal ligament of perineum in the cavity of small basin, where runs into prostate veniplex. At presence of two trunks, they run into prostate veniplex independently.

We, having carried out the research of the venous channel of the penis of 160 corpses [10], in one of them we have not found

Figure 2. **Veins of the penis of man (corrosion preparation): 1 – retroglandular veniplex; 2 – deep dorsal vein**

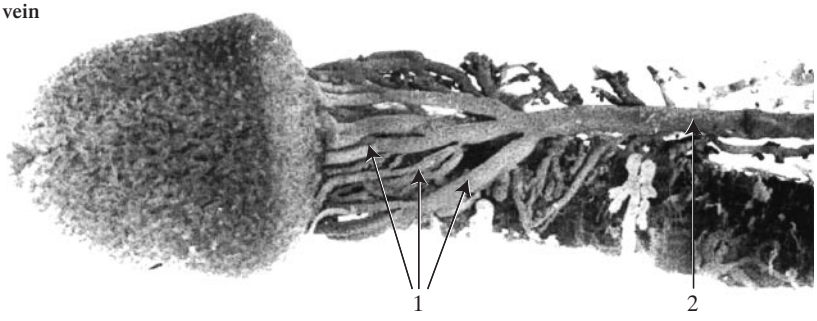
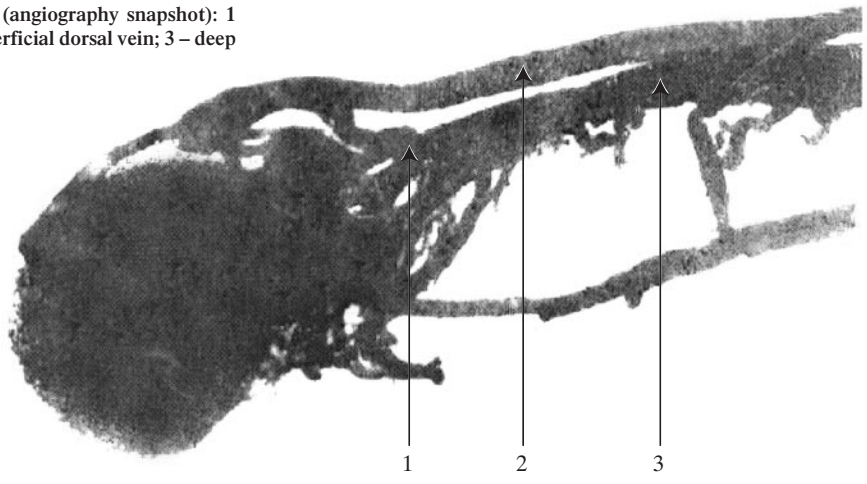


Figure 3. **Veins of the penis of man (angiography snapshot): 1 – anastomosis between veins; 2 – superficial dorsal vein; 3 – deep dorsal vein**

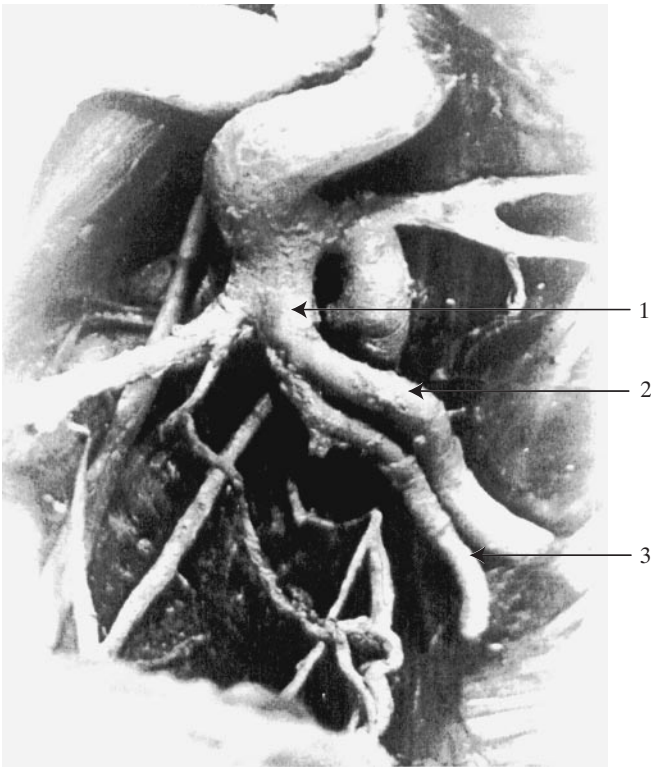


deep veins of the organ. According to it we consider that the deep veins of the penis as real anatomical object in the norm are absent. The rather low efficiency of the method of surgical correction of vascular impotency of venous genesis offered the Brazilian scientists bears also to it [11]. They assert, that the deep veins of the organ act from a proximal part of cavernous bodies at once after place of their bifurcation, and the nature of their operation consists that the cavernous bodies in their proximal part, i.e. pinch of penis dressed completely.

The spongy veins execute the venous outflow from a like body. As a rule, it is a pair veins (93.7%) with the average diameter of 0.87 ± 0.01 mm located on the lateral area of the spongy body under the deep fascia of the penis. They are formed from small-sized venous vessels leaving in the area of urethral sulcus. The spongy veins outstanding as third venous collector, reach the bulb of the penis, where they receive small-sized bulbar veins. Then pass the interval between the arcuate ligament of the pubis and the transversal ligament of the perineum and run into the prostate veniplex (88%). In some cases, (12%) they run into the deep dorsal vein in the field of the perineum.

The arterial blood supply of the penis takes place at the expense of external and internal pudendal arteries [12]. The external pudendal artery starts from the internal wall of the femoral artery 2.5-2.7 cm below the inguinal ligament. In some cases (8%) the artery starts by two trunks – forward and back. The diameter of the initial department of the external pudendal artery or its forward trunk varies from 1.0 up to 2.1 mm. The external pudendal artery, and in the case of it the forward branch division, goes ahead to the femoral vein below the places of locking in last of large hypodermic vein of the femur. In the region of the hypodermic slot of the femur, the artery perforate the loosened site of the broad fascia of femur also passes in hypodermic fat, being routed in tracking of the similar vein to the upper edge of the body of the pubic bone. At level of the pudendal artery returns last a branch with average diameter of 0.4 ± 0.01 mm to a skin of the scrotum. The external pudendal artery with average diameter of 0.95 ± 0.02 mm goes to the basis of penis, where it turns under a corner in the party of the head of the penis and is located above the deep fascia of the organ. It is necessary to note, that the indicated branch of the external pudendal artery goes parallel dorsal artery of the penis also passes from it to the left apart 0.25 ± 0.01 mm, and on the right – 0.2 ± 0.01 mm. Thus, the branch of the dextral external artery intersects the dextral dorsal artery at the level of mean third of body and is located internal from it. At the same time, as a branch of the left external pudendal artery, which reaches for the left dorsal artery, in our material never intersected her and is situated external that matters at operations on arteries of penis. The diameter of final department of the external pudendal artery on the average makes 0.68 ± 0.02 mm. The back of the trunk of the external pudendal artery, whose average diameter makes 0.8 ± 0.01 mm, as against the upper trunk places behind the femoral vein and at once breaks up to small-sized veins in a skin of scrotum. It is necessary to note the fact, that the

***Figure 4*. Arteries of the pelvis of man: 1 – common trunk for inferior gluteal and internal pudendal arteries; 2 – inferior gluteal artery; 3 – internal pudendal artery**



dextral external pudendal artery in its proximal 2/3 always has the main type of the constitution, in too time as the left external pudendal artery in 12,5% of cases has loose the type, when after waste from femoral artery it is divided into small-sized branches going to penis, to scrotum, to skin of the femur and the forward abdominal wall.

The internal pudendal artery is the main source of the blood supply of the penis of a man [12,13]. It moves from the forward trunk of the internal iliac artery independently in 50% of cases. In other cases it either moves from this artery by one trunk (*Fig. 4*) with lower gluteal (36%), common trunk with the upper and lower gluteal arteries (4%), or with upper gluteal (8%), or with obturator artery (2%). The internal pudendal artery with average diameter of 3.6 ± 0.02 mm leaves the cavity of a small basin through the infrapiriform opening. Then bends the ischial spine and through the small sciatic foramen catches in the cavity of ischiorectal fossa. According our data, the internal pudendal vessels and the accompanying nerve are situated on the lateral wall of the ischiorectal fossa. In this place, they are concluded in special fascial shell formative pudendal channel. The fascial shell accretes with the bottom of the obturator fascia. Being spread upwards, it accretes with the lower fascia of the pelvic diaphragm and, being spread downwards, passes on the falculate process of ischio-tuberal ligament. In 100% of cases, the internal pudendal artery has a turnpike – a free type of constitution. The overall length makes it from infrapiriform opening before branching 94.3 ± 0.03 mm. Back or ischiorectal department of the internal pudendal artery with the average diameter of 3.3 ± 0.03 mm deposits on depth from the top of the tuber of the ischium 66.4 ± 0.02 mm. The forward or the genitourinary department of the internal pudendal artery on caliber is a little bit less 2.8 (0.01 mm) places on depth 63.2 ± 0.04 mm from the top of the tuber of the ischium. The lower rectal artery with the average diameter of 1.58 ± 0.02 mm and the length of 37.8 ± 0.03 mm starts from the internal pudendal artery at a level of ischial tuber. The artery of the perineum with the average diameter of 1.2 ± 0.01 mm and the length of 1.7 ± 0.02 mm removes a little bit below. After deriving the artery of the perineum the internal pudendal artery has a title of artery of penis; it is posed in horizontal plane between pelvic and back department of the genitourinary diaphragm, then at a level of the bulb of the penis, perforate the genitourinary diaphragm is returned by two branches: bulbar and urethral arteries. The bulbar arteries with the average diameter of 1.6 ± 0.02 mm enter the bulb of the penis. Here they return reflexive branches, blood supply the given department of the penis, and, gradually made by thin, follow forward in spongy body and in mean third they are bound with branches of urethral arteries. Urethral artery with the average diameter of 1.73 ± 0.03 mm removes from artery of the penis 0.5 cm distal from bulb. They in pour into spongy body at the place of connection it with the cavernous bodies and pass through the spongy body in longitudinal direction, returning on the stretch short branches in its material, and it's bound with branches dorsal arteries of the penis.

The arteries of the penis with the average diameter of 2.3 ± 0.03 mm after about passage of it bulbar and urethral arteries under pubic symphysis are divided into the final branches: deep and dorsal arteries of the penis. The dorsal artery with the average diameter of 1.9 ± 0.02 mm takes the lateral position on the inferior surface of the deep transversal muscle of the perineum. The initial department of the dorsal artery of the penis is covered with the lower fascia of the genitourinary diaphragm. For forward boundary of the genitourinary triangle artery is covered with a tendon of ischiocavernous muscle, which in place of transition of the artery on the dorsum penis is fixed strongly by vessel to the inferior surface of the pubic bone and to the ligament, executing the pubic corner. Dextral and left dorsal arteries are located under deep fascia of the penis on the dorsum of penis. At the initial department of the organ the arteries run parallel to the dorsal vein and lateral from them place dorsal nerves. In forward third of body of penis dorsal arteries displace on inferiolateral surface of cavernous bodies, i.e. dorsal nerves will lie here from the medial party. In a place of decussating, the dorsal artery places superficially from dorsal nerve. From dorsal arteries 5-6 pairs of the circumflex arteries with the average diameter of 1.1 ± 0.03 mm starts. They sequentially go out cavernous bodies of the penis and on lower – lateral area they perforate dense white. In more thickly cavernous tissue they are bound with branches of the deep arteries of the organ. Besides it is necessary to note, that up to perforation of white dorsal arteries anastomose also with urethral arteries. Both dorsal arteries, having entered in the head of the penis, decrease in diameter and, incorporating among themselves and final bifurcations of the deep and urethral arteries, will derivate extensive anastomosis, from which in various directions miss are numerous divided branches. Incorporating among themselves for the type of arcade, they will derivate a peculiar arterial framework. The deep arteries with the average diameter of 1.83 ± 0.02 mm enter penis in the field of the medial surfaces of its pinches. In cavernous body the deep artery goes in longitudinal direction, taking position is closer to the dividing wall. On all stretch from it the numerous cochlear arteries move, which quantity gradually decreases in the direction of the head. In distal and mean third of the organ, the deep arteries well to are bound among themselves. In same area arteriolo-venular shunts between branches of the deep artery and dorsal vein of the penis exist. The main trunk of the deep artery, as a rule, in a mean third has sphincters, which represent the thickening of muscular coat of wall of the artery.

In findings of investigation is established, that the venous outflow from the penis is realizes on three veins: the superficial dorsal, the deep dorsal and the spongy vein. The arterial blood supply of the penis take place at the expense of external pudendal arteries, bulbar, urethral, dorsal and deep arteries of the penis. The head of the penis represents the system of the anastomosis between urethral, dorsal and deep arteries of the organ. The constitution of the circulatory channel of the penis has a number of features, which must be taken into account to realize the surgical correction of the erectile vascular impotency.

Discussion

The given research is dedicated to analysis of variant anatomy of the vascular channel of the penis.

Our research demonstrates, that though the intraorgan arterial channel of the penis is partitioned on different departments having its own morphological features, functionally it is the unified system, as all intraorgan arteries are wide anastomoses among themselves. Apart from numerous anastomoses, between same name arteries of both sides the following constant intersystem anastomoses connecting different arteries are present:

1) Arterial arch of the head of the penis, where dorsal, deep and urethral arteries of both sides anastomose;
2) Arterial plexus of the urethral sulcus, in formation which the branches of the dorsal, deep and urethral arteries participate;
3) Anastomosis between dorsal and deep arteries in the tissues of the cavernous bodies itself.

Therefore confirmations of some researchers that "urethral arteries vascularise only bulbus of the penis [14], or that the dorsal arteries vascularise head of the organ [11], or that the main source of influx of the arterial blood in cavernous bodies are the deep arteries" [10] are not absolutely precise. According our data, all three arteries (dorsal, deep and urethral) almost equally participate in the blood supply of the penis. The dorsal arteries as well as deep execute of influx of the blood in cavernous bodies through the spiral arteries, which are vessels of the muscle-elastic type, i.e. with high functional activity. The presence of a longitudinal layer of muscle cells situated between the endothelium and an internal elastic membrane, is that apparatus, with the help of which the lumen of the spiral arteries change. The spiral course of longitudinal muscle cells results that in torsion, to elongate of all system, changes diameter of the lumen and regulates the blood stream. Thus, for valuable influx of the blood in cavernous bodies two pair of arteries should operate.

During research of the venous system of the penis series of features have found which either are not described in the literature, or it is required refinements. The division of the deep dorsal vein on two trunks has direct practical value. As a rule, the surgeon dresses or resects the trunk of the deep dorsal vein, which places in an intercavernous sulcus between dorsal arteries and nerves. If there are two trunks, one of them lies between dorsal arteries, and another places externally from these vessels and during operation is not affected by the surgeon that, apparently, has an effect of correction of the venous outflow.

Divergences with literary data are available also in the attitude of sources, number and basic dislocation of the circumflex veins. The some authors [15] consider that in norm there are two-three pairs of the circumflex veins locating only in distal one third of the penis, and consider as the sign of infringement of the venous hemodynamics their greater number and their other localization. On data of our investigation, it was seen more often four or five pairs of the circumflex veins drain in deep dorsal vein (but not in superficial dorsal vein, as affirm some authors [16]), and on the right them is localized a little bit more, than at the left.

We have found the circumflex vein, which drained the blood from the distal parts of cavernous bodies, and ran into deep dorsal vein behind its valve. Description of this vein in the accessible literature we have not met. The ignorance by the surgeon of this fact can have an effect on results of surgical treatment of the erectile dysfunction of the venous genesis at the ligation or resection of the deep dorsal vein.

As it was mentioned above, we affirm, that the deep vein of the penis misses: venous outflow from a most part of organ is carried out by the deep dorsal vein, and the proximal parts of cavernous bodies are drained at the expense of veins of the crura of the penis. But Hodos AB (1963) described the deep vein as the unpaired vessel, which passes from the head up to the root on the dorsal surface of the spongiform body in the urethral sulcus. On a transit the vein forms sine dilatings, from which circumflex veins originate. Series of other authors [16,17] guess, that the blood from the central part of cavernous bodies flows off in the deep vein through short postcavernous veins collecting blood from cavernous spaces and from venules, posed inside of cavernous tissue. The direct outflow of the blood was postulated on the basis of "locked valves" in deep veins in mammals, having osteal basis in the penis and found in experiment. But in cavernous bodies of the penis of the man there is no such structure. Exponential is that documentary any author does not confirm presence of the deep vein of the penis. Therefore should be logical, radiating from function of the penis, that the reinforced inflow is important first of all, at the expense of which penis acquires an erection, and the retarded outflow from cavernous bodies. For this reason, deep dorsal vein is accompanied with two dorsal arteries, but not opposite.

The venous channel of the penis cannot be surveyed as separately taken veins, as it represents a uniform extensive complex of anastomoses. For example, assertion that the spongiform veins realize outflow from the same (spongiform) bodies is a little bit untrue, as due to their anastomosis with circumflex veins, they can be main structures in the drainage of the blood from cavernous bodies. The similar situation is with superficial dorsal veins, which are in most cases connected by means of intervenous anastomosis with deep dorsal vein.

Conclusions

In conclusion, during of investigation it was fixed, that the venous outflow from the penis is carried out on three veins: superficial dorsal, deep dorsal and spongiform veins. The external pudendal, bulbar, urethral, dorsal and deep arteries of the penis take part in arterial blood supply of this organ. The head of the penis represents system of anastomoses between urethral, dorsal and deep arteries. The structure of the circulatory channel of the penis has series of features, which are necessary for taking into account at performing of surgical correction at an erectile vascular dysfunction.

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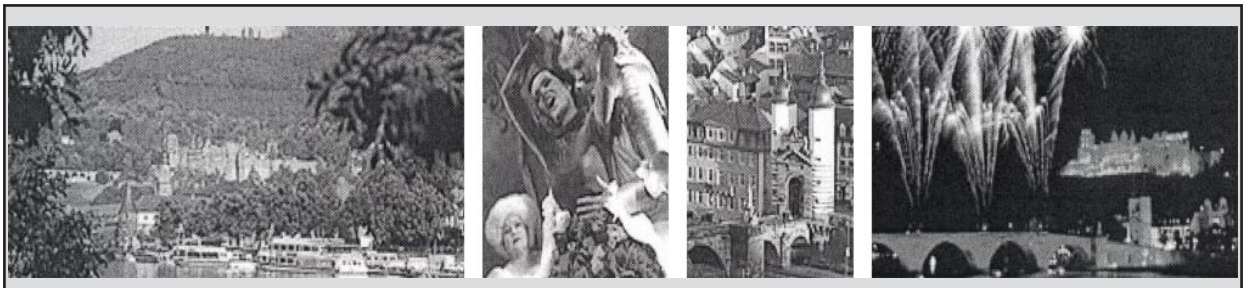

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