# Evaluation of secretory mucin concentration of patients with squamous cell carcinoma oral cavity

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## Abstract

**Purpose:** **Secretory salivary mucins constitute a heterogenous group of glycoproteins, synthesized and secreted by submandibular, sublingual gland and small glands of oral mucosa. The most significant functions of mucins in case of oral cavity carcinoma are: participation in oral pellicle formation, lubrication and creation of heterotypic complexing.**

**The aim of this study was to assess mucins concentration, and finally to establish the correlation between concentration of mucins in saliva and clinical advancement according to TNM.**

**Material and methods:** **The research was conducted on mixed resting and stimulated saliva of patients with oral squamous cell carcinoma. Mucin's concentration was measured one day before, and thirty days after surgical procedure. The volume of saliva was volumetrically determined, quantitative evaluation of mucins was accomplished by PAS method.**

**Results:** **In comparison with K group, a significant decrease of mucins was found in resting and stimulated saliva of patients with carcinoma in all degrees of clinical advancement. Mean value of mucin in resting and stimulated saliva after surgical treatment were lowered. The degree of carcinoma clinical advancement correlated negatively with mucin concentration.**

**Conclusions:** **The decrease of mucin contained in saliva may be important in further evolution or progression of carcinoma. The results also suggest that saliva may be a significant diagnostic material in carcinoma research.**

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## Introduction

The oral mucosa is constantly exposed to the effect of damaging factors (physical, chemical, biological). In normal conditions, the effects of these factors are counteracted by many interconnected protective mechanisms of the oral cavity. They include: anatomical integrity of the mucosa, the presence of saliva, its constant flow, as well as protective and regenerative substances it contains.

The correct flow of saliva enables the formation of a protective layer covering the oral mucosa, limiting the penetration of potentially carcinogenic compounds into the epithelium. Mucins are among the agents participating in the formation of the architectural skeleton of the protective preepithelial layer in the oral mucosa, as well as maintaining correct concentrations of protective substances.

Mucins contained in human saliva represent a heterogenous group of glycoproteins synthesized and released by the submandibular and sublingual glands, and small glands of the oral mucosa [1,2]. Currently, two main groups of salivary mucins are identified: a) high-molecular MG1 mucins of molecular mass above 1 000 kDa, b) low-molecular MG2 mucins of molecular mass 200-300 kDa [3].

High viscosity of mucin, significantly affecting its separation from the other organic components of saliva, indirectly shows the ability of mucin molecules to form complex connections with other types of molecules. These connections are referred to as heterotypic complexes in which mucin molecules may selectively bond with the other organic substances in saliva such as: IgA, lysozyme and lipids [4]. This type of intermolecular interactions involves ionic and hydrogen chemical bonds, as well as hydrophobic interactions. Due to the formation of heterotypic complexes, salivary mucins may play the role of carrier of substances they are bound to. The shown ability of MG1 and MG2 to form heterotypic complexes may have an effect on the

*Table 1*. **Clinical stage**

| Clinical stage | Number of patients | Percentage |
|---|---|---|
| S1 | 6 | 12.50 % |
| S2 | 8 | 16.70 % |
| S3 | 12 | 25.0% |
| S4 | 22 | 45.83% |

*Table 2*. **Carcinoma location**

| Location | Number of patients | Percentage |
|---|---|---|
| tongue | 10 | 20.83 % |
| tongue + oral cavity floor | 15 | 31.25 % |
| oral cavity floor | 9 | 18.75 % |
| soft palate | 5 | 10.42 % |
| buccal oral mucosa | 6 | 12.50 % |
| inferior gingiva | 3 | 6.25 % |

increase in concentrations of protective substances in the layer of mucus covering the surface of the organs and tissues of the oral cavity [5].

Saliva and the protective agents it contains have been evaluated in patients with oral cancer in a few clinical trials involving small groups of subjects. The role of protective factors in saliva, including mucins, in the biology of the development of planoepithelial carcinoma of the oral cavity has not been determined so far.

Taking into account the above data, a study of resting and stimulated mixed saliva in patients with squamous cell carcinoma of the oral mucosa before and after surgery was undertaken. The purpose of the study was to determine mucin concentrations, and to establish correlation between its concentrations and clinical stage of tumour according to TNM classification.

## Material and methods

The study material consisted of resting and stimulated mixed saliva from 48 patients with histopathologically verified planoepithelial carcinoma of the oral cavity. The age in the study population was 39 to 80 years (mean age 60 years). There were 39 men and 9 women among the patients.

To evaluate clinical stage of tumour, the four-degree scale according to TNM classification (fifth version) [6] was used:

I stage ( S1) – T1, N0, M0
II stage (S2) – T2, N0, M0
III stage (S3) – T1, N1, M0 ; T2, N1, M0 ; T3, N0, M0 ; T3, N1, M0
IV stage (S4) – T4, N0, M0 ; T4, N1, M0 ; Each T, N, M1

The patients most often had tumours in clinical stage IV (45.83%), and less often in stage I (12.5 %) (*Tab. 1*).

The most frequent location of tumour was the tongue with the oral fundus (31.25%), then the tongue (20.83%), and then the oral cavity floor (18.75%) (*Tab. 2*).

The exclusion criteria were diseases in which saliva production is impaired (diabetes, Sjögren syndrome), or the use of pharmaceuticals affecting saliva production. All participating patients and all subjects in the control group were smokers (20-30 cigarettes a day on the average, for a period of about 20 years). The results were compared to the control group consisting of 25 healthy people (mean age 58 years).

*Table 3*. **Mucin concentration (mg/ml) in resting saliva of patients before surgical treatment. (S1–S4) and K group**

| | K | S1 | S2 | S3 | S4 |
|---|---|---|---|---|---|
| mean value | 0.88 | 0.79 | 0.68 | 0.52 | 0.43 |
| SE | 0.11 | 0.18 | 0.17 | 0.10 | 0.15 |
| n | 25 | 6 | 8 | 12 | 22 |
| coefficient value (p) | | $p > 0.05$ | $p > 0.05$ | <0.001 vs K | <0.001 vs K |

In both patients and healthy subjects, saliva was taken using the spitting method, in 10-minute fractions (the first fractions were taken without secretory stimulation of the salivary glands, and the other fractions were taken upon saliva secretion stimulation by parafilm chewing). Until the time of assays, the material was stored at -80°C [7]. Saliva was taken from the patients 1 day before and 30 days after tumour excision surgery.

Data distribution was analysed with Shapiro-Wilk test. The subsequent hypotheses were tested using t-Student test, and the results were presented as arithmetic mean ±SE (standard error). The results for which the coefficient value was $p<0.05$ were considered statistically significant. To evaluate the mutual relationships between mean mucin concentrations and the clinical stage of tumour, Pearson linear correlation test was used. The correlation was considered complete for the coefficient values of $r\geq0.9$ [8].

A quantitative evaluation of mucin in saliva was performed based on the PAS method (periodic acid/Schiff reagent) described by Mantle et al. [9].

The subjects expressed written consent for saliva sampling, declaring informed participation in the clinical study. The above study was approved by the Bioethics Committee at the Medical University of Białystok (35/2000).

## Results

In healthy people (K), mean mucin concentrations in resting saliva were at the level of 0.88 mg/ml. Compared to the K group, presurgery mean concentrations of mucin were reduced in the patients participating in the study (S1–S4). The lowest mucin concentrations were found in saliva of patients in clinical stage S4, and the highest – in stage S1. Compared to the control group, this reduction was only statistically significant in patients in disease stages S3, S4 ($p<0.001$) (*Tab. 3*).

In all patients, mean mucin concentrations in resting saliva became further reduced after surgical treatment. The observed differences in mucin concentrations in resting saliva of patients before and after tumour excision were only statistically significant in patients in stages S3, S4 ($p<0.05$) (*Fig. 1*).

*Figure 1*. **Mucin concentration in resting saliva of patients before and after surgical treatment**

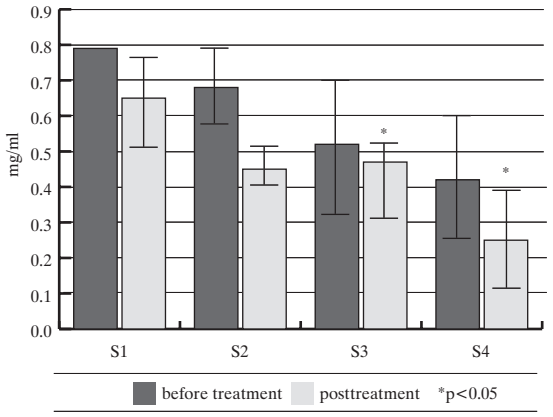



*Figure 2*. **Mucin concentration in resting and stimulating saliva of patients before surgical treatment and K group**

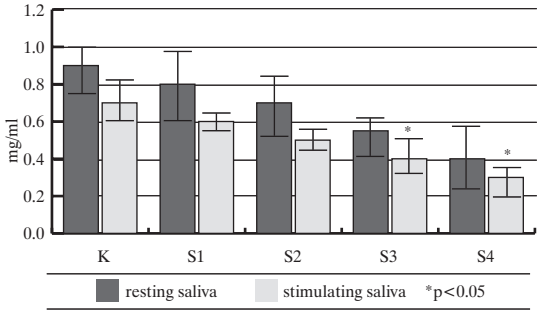
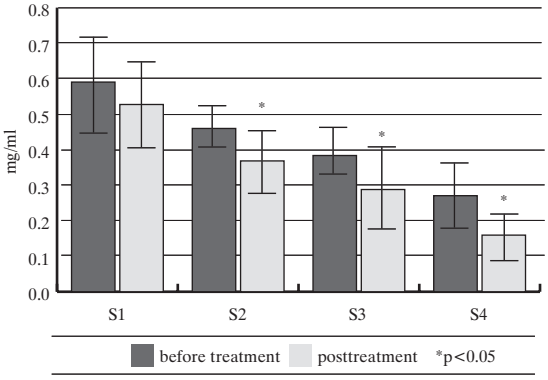



*Figure 3*. **Mucin concentration in stimulated saliva before and after surgical treatment**




Upon secretion stimulation in subjects from the K group, a reduction in saliva mucin concentrations was seen compared to concentrations achieved with resting secretion (0.71 mg/ml). The reduction was not significant ($p>0.05$). Also, in all patients (S1–S4), before surgery, a reduction in mucin concentrations in stimulated saliva was seen compared to the resting saliva concentrations. This glycoprotein reduction was statistically significant in patients in disease stages S3 and S4 ($p<0.05$) (*Fig. 2*).

In all patients (S1–S4), mean mucin concentrations in both stimulated and resting saliva were further reduced after surgical treatment.

The observed differences in mucin concentrations in stimulated saliva before and after tumour excision were statistically significant in patients in disease stages S2, S3, S4 ($p<0.05$) (*Fig. 3*).

Searching for mutual relationships between mean mucin concentration in resting and stimulated saliva in patients before and after surgery, and the clinical stage of tumour, a slight trend towards negative correlation was found $r=(-0.5)$.

## Discussion

On the surface of mucosal epithelial cells, there are high-molecular glycoproteins referred to as mucins. Due to the variety of their functions and structure, they are divided into membrane mucins, forming part of the cellular membrane, and secretory mucins which are the primary component of mucus [10-12]. The significance of secretory mucins present in saliva in non-immune protective mechanisms has been well documented for the teeth [12,13]. However, little is known about the role of these glycoproteins in maintaining the integrity of the oral mucosa.

In the present study, an evaluation of mucins in resting and stimulated mixed saliva was performed in 48 patients with oral cancer in various clinical stages of tumour. The results were compared to a control group.

Before surgery, all patients had lower mucin concentrations in resting and stimulated saliva compared to the group of healthy subjects. The concentration values of this glycoprotein were reduced with the increase in clinical stage of tumour. It was also confirmed by the observed trend towards negative correlation. The results of the authors' own studies have shown that in patients with oral cancer, the ability of the salivary glands to secrete mucin is limited.

In the literature, there are no studies evaluating secretory mucin concentrations in saliva of patients with oral cancer.

The evaluation of minute secretion of saliva before surgery shows normal function of the salivary glands in the study subjects in qualitative aspect of produced and secreted saliva [14]. However, it does not rule out the possibility of impaired quality of saliva components produced. Eliasson et al. showed a reduction in mucins and other salivary components in smokers. Assessing the morphology of the palatine glands (mixed, prevalence of mucous cells), they showed a dilation of external ducts with excessive mucus retention, as well as atrophy of the acinic cells. They also observed the presence of mucus and inflammatory cell clusters in the interstitium. These authors link the reported changes with the vasoconstrictor effect of the products of tobacco smoking and the subsequent reduction in blood flow through the glands, and the change in qualitative composition of saliva produced, including a reduction in mucins [15].

The subjects in our study were long-term heavy smokers. Taking into account this fact and the results of the cited studies, it may be assumed that mucin reduction in patients with oral cancer may be caused by similar morphological changes in sub-

lingual and submandibular glands which are the main source of secretory mucins in saliva. However, in the group of the patients participating in the study, it is difficult to distinguish the results of using nicotine from the possible effect of tumour mass compressing the submandibular and/or sublingual glands. It should be emphasised that most tumours located in the fundus of the oral cavity and in the ventral surface of the tongue are in disease stages S3–S4. It is possible that mechanical effect of tumour by compression may also be a cause of local reduction in blood flow in the glands, and as a consequence, a reduction in secretory mucin concentrations.

It may therefore be assumed that as a result of reduction in mucin concentrations in saliva of patients, the mucosa may be more susceptible to the effect of damaging factors which, with long-term exposure, may lead to initiation, promotion and/or progression of the carcinogenesis process.

The question is whether the reduction in mucin concentrations in saliva of patients is primary or secondary to the neoplastic process? The studies carried out in the last ten years by various authors [2,4,16-18] emphasise that mucins which are the main component of the preepithelial protective layer of the mucosa not only represent a mechanical barrier but also a dynamic structure modelling the oral cavity environment.

Specific rheological properties of salivary mucins contribute to the formation of a thin layer covering all structures in the oral cavity. A strong affinity of mucins to the gastrointestinal, respiratory and genital epithelium has been accepted since long ago as the prerequisite for film formation on the oral surfaces. The stability of these interactions was described as various hydrophobic and ionic bonds between mucins and the surface of the mucosa [19-21].

Moreover, Słomiany et al. [17] showed that mucins in the area of the gastric mucosa bind to a specific membrane receptor. Similar receptors were also identified and described in the epithelial cells of the buccal mucosa. It was shown that the mucin-receptor bond requires presentation of oligosaccharide chains of mucins by breaking some β-glycosidic bonds inside the chains. It evidences the dynamic character of these interactions.

The studies of Murty et al. showed that the breakage of mucin-receptor bonds by bacterial glycosidases may lead to a loss of pre-epithelial barrier of the oral epithelium. When unprotected against the effects of exogenous factors (including carcinogens), the mucosa becomes susceptible to ulceration and further progression of lesions towards cancer [17,22]. It is an important observation in the aspect of carcinogenesis of the oral mucosa, because the factors determining the promotion and progression of cancer process may include chronic inflammation, as well as poor oral hygiene [23,24]. The presence of bacteria in the saliva, accompanying the described changes, may be associated with a loss or impairment of the mucin coating, and increased penetration of possible carcinogens into the epithelium.

Based on the morphological criterion, three compartments of protective action have been identified in the oral mucosa: preepithelial, epithelial, and postepithelial compartments.

Due to the fact that damaging factors act from the lumen of the gastrointestinal tract, the key importance is attached to the preepithelial barrier. The correct functioning of this barrier mostly relies on mucin and non-mucin proteins [25]. The protective effect of mucin depends on its ability to form an architectural skeleton inside the barrier, responsible for the inhibition of diffusion of damaging factors. As the barrier is highly hydrophobic and contains many phospholipids, it may bind other protective factors such as: epithelial growth factor (EGF), prostaglandins (PGE2) [2,4,18,26]. The thickness of the barrier is 0.05 to 0.1 mm, and direct dependence between its thickness and the protective properties was shown.

The ability to form heterotypic complexes with non-mucin proteins including sIgA and lysozyme [20], as well as EGF and PGE2 [16] also highly determines the functions of mucins in the oral cavity. A reduction in mucin concentrations in saliva of our patients may, therefore, be the cause of reduction in concentrations of many protective factors in the mucinous layer, and as a consequence, in the epithelium of the mucosa. The reduced protective potential of the epithelium in patients with S3–S4 stage may therefore exacerbate the existing lesions in the tumour but also in other parts of the mucosa, and in the upper gastrointestinal tract. Recent studies have also shown that mucins, apart from forming the described mechanical protective layer and the formation of heterotypic complexes, may also modulate/regulate intramembranous mechanisms such as regulation of intracellular calcium levels, related to the function of various growth factor receptors [27]. The results of studies performed in the latest years suggest that disorders of so-called calcium transmitter system [22] underlie the "chemical oncogenesis" occurring in the oral cavity. Calcium ions belong to the group of so-called secondary cell transmitters and represent an important element regulating many functions of the cell. The secondary transmitter system multiplies the signal in the cell [28].

The study of Knaus et al. showed that the function of calcium channels depends on polyanionic molecules, e.g. heparin and GM1-ganglioside. Peppelenbosch et al. [29], Słomiany et al. [17] found that the process of phosphorylation of these channels is a response to the effect of growth factors. Later studies of Słomiany et al. of calcium channels in the buccal mucosa showed that mucins (both low- and high-molecular) may also modulate their activity in the soft tissues of the oral cavity. Subsequently, it was found that the acidic fractions of mucins have an inhibitory effect against calcium channels. It was shown that this effect was related to the presentation of sialic acid and ester sulphone groups in the oligosaccharide chains of mucins [17,30].

Słomiany et al. [17] presented the inhibitory effect of mucins against calcium channels using the example of EGF. EGF, by binding to a membrane receptor connected with a calcium channel, causes its phosphorylation, activation of tyrosine kinase, leading to calcium channel opening and an increase in calcium ion concentrations in the intracellular environment. In these conditions, EGF bound to salivary mucins does not bind to membrane receptors; as a result, a reduction in calcium ion concentrations in the intracellular environment occurs.

The possibility of calcium channel activity modulation by mucins is, therefore, another property contributing to the multifunctionality of salivary mucins in the aspect of oral mucosa protection.

At present, many authors agree that disorders of transmission of signal received by EGFR have a significant effect on the basic function of the cell controlled by this receptor, i.e. differ-

entiation, maturation, proliferation, adhesion, migration and apoptosis inhibition [31,32]. Sometimes the disorders of the pathways of signal transmission from EGFR to the cell nucleus are also manifested as promotion of cancer transformation and tumour proliferation, an increase in invasiveness of its cells and cell survival, as well as in the form of neoangiogenesis.

Taking into consideration these data from my studies, a reduction in mucin concentrations in patients with oral cancer may directly affect the functioning of various membrane receptors of growth factors with tyrosine kinase activity, thus promoting the carcinogenesis process in the entire area of carcinogenesis.

In our studies, mucin concentrations in patients after surgery were also lower than in the group of healthy subjects. These differences were significant for patients with disease stages 3 and 4. However, an analysis of mucin concentrations in resting and stimulated saliva in the group of patients before and after surgery showed a significant reduction of this glycoprotein after tumour excision.

In physiological conditions, salivary mucins are synthesized by the salivary cells of the sublingual glands (60% of mucous cells) and submandibular gland (5% of mucous cells), as well as small salivary glands located in the palatine, buccal and labial mucosa. On the contrary, the parotid glands represent the type of serous glands and the mucous cells are rarely seen in their structure [33]. It has been shown in both an animal model and in humans that these glands do not participate in the production of salivary mucins.

Taking into account the above data, further reduction in mucins in both fractions of the saliva after surgery, seen in the group of patients, may be related with the removal of submandibular and sublingual glands during surgery.

The reduction in mucin concentrations in the saliva in patients with cancer due to impairment of the preepithelial barrier may have an effect of the progression of tumour growth, and further reduction in their levels after surgery may be responsible for the occurrence of local relapse. As resting saliva represents the oral environment for a significant part of the day (14 to 16 h) [34], mucin concentrations in this fraction of saliva seems particularly important.

To recapitulate, it should be concluded that a reduction in secretory mucins in both fractions of saliva in patients with oral cancer, and a trend towards negative correlation with the clinical stage of disease, may indicate functional impairment of the preepithelial barrier of the mucosa in these patients. However, the issue requires further investigation.

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