Molecular examination of the microenvironment of head and neck tumors

PhD Thesis

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1. Introduction

More than 90% of head and neck cancers are squamous cell cancers with a 5-year survival rate of only around 60%. Tumors are often diagnosed in an advanced stage due to the lack of specific symptoms and diagnostic difficulties. The more advanced the stage of the tumor, the more invasive the diagnostic and therapeutic interventions required. Therefore, early molecular diagnosis could be of pivotal importance primarily with respect to increasing the survival rate. Recent decades failed to see an improvement in survival rates despite development in diagnostic and therapeutic methods.

The aims of the thesis were originated from these data – a more widely-available method would be needed which could enable early diagnosis of the tumors and would also be suitable for follow-up.

1.1. Epidemiology of head and neck tumors

In Hungary, out of the six most prominent groups of mortaliy causes, cancer deaths come second after cardiovascular diseases taking the first place. According to European mortality statistics, Hungarian men are first, whereas Hungarian women are the second regarding cancer mortality. According to data from the Hungarian Central Statistical Office, mortality of cancers of the upper aerodigestive tract has increased markedly since the 1970s, mainly, due to malignant processes affecting the lips and oral cavity. The number of these cancers has trembled and their mortality has become nearly five-fold.

The mortality of head and neck cancers has changed significantly in the past 50 years, in Europe the mortality rate has doubled. While in Hungary all-cancer mortality has increased 2.8 fold during the period between 1948 and 2000, head and neck cancer mortality showed a six-fold increase. Data demonstrate that it is head and neck cancer mortality that shows the most dynamically increasing tendency in Hungary, putting us in the first place in Europe.

Since the 1970s, mortality has increased nearly six-fold, whereas in Europe it has only doubled, thus Hungary is in a ’leading’ position among European countries.

1.2. Aetiology and risk factors

Regarding the aetiological risk factors influencing the development of head and neck cancers, excessive alcohol consumption and smoking, behavioral factors that could theoretically be changed, are to be emphasised.

1.2.1. Smoking

The most important causative factor in the background is smoking. Regarding carcinogenesis, it is not only smoking cigarettes that is important, smoking is equally harmful with or without direct inhalation of smoke. Passive smoking increases the incidence of cancers of the respiratory tract two-fold among those who share a home with a smoker.

In Hungary, nearly one third of the population over age 15 and older, that is more than 2.5 million people smoke, most of them on a regular basis. Sudden cessation of smoking only brings results slowly, it does not influence being in the high risk category for at least three years, it results in a gradual decrease only thereafter. A smoker will reach the ’non-smoker’ category in terms of cancer risk only after approx. 10-15 years.

1.2.2. Alcohol consumption

Another significant aetiological factor is alcohol consumption, in any of its forms. Alcohol consumption habits characteristic of Hungary facilitate cancer development. Besides the amounts consumed, the quality of the alcohol is also of great importance: the consumption of spirits characteristic in our country is especially harmful in this respect. The coexistence of these two aetiological factors increases the risk of head and neck cancers 15-fold, as etanol promotes the absorption of the carcinogenic components of cigarette smoke.

1.2.3. Inadequate oral hygiene

Inadequate oral hygiene in itself is a mechanical irritative factor, thus can cause chronic inflammation or even precancerous states. Moreover, the acetildehyde produced by the microflora of the plaque has a direct chemical carcinogenic effect. Inadequate oral hygiene has been proven to increase the incidence of oral HPV infections.

1.2.4. Infections

The DNA of HPV can be detected in 40-60% of oral and oropharyngeal tumors. In more than 95% of the cases, a high-malignancy P16 serotype is found in tumors of the tongue base or tonsillo-lingual region. According to other studies, the separate or concommittant presence of EBV and JCV can also be associated with oral cavity cancers of epithelial origin.

1.2.5. Malnutrition, inadequate diet

Malabsorption can be in the background of cancers of the oral cavity in approx. 10-15 % of the cases.

1.2.6. Immunosuppressed status

An impaired immune system may also lead to the development of oral cavity cancers.

1.2.7. Age and sex

A few years ago, head and neck sqamous cell carcinomas occured mainly above age 40 in Europe and also in Hungary, however, previous years have seen an increase in younger ages as well. Differences between the sexes can be partly due to differences in the frequency of addictive factors and partly to differences in life style habits.

1.2.8. External factors, occupation

Exposure to ionising radiation, air pollution, substandard social environment and stress may also play a role in carcinogenesis. Patients' visiting their doctors late, at more advanced stages of the disease, also contributes to worse chances of survival.

1.2.9. Endogenous, genetic factors

The status of the individual organism also contributes significantly to the manifestation

of the impact of environmental factors, this individual predisposition is largely determined by endogenous, genetic factors.

1.3. The significance of early diagnosis and treatment

One of the main reasons for the high mortality of head and neck tumors could be the fact that the majority of patients are diagnosed at an already advanced stage of the disease, when therapeutic possibilites are apparently more limited. As there are no symptoms specifically characteristic of head and neck tumors, these tumors often remain „hidden" for a long time and can often only be distinguished from an other 'simple' condition based on the long duration of the symptoms.

When diagnosed early, however, and if there are no metastases in the neck, surgery and radiation therapy usually provide excellent long-term results. However, most patients do not fall into this category, most cases require extensive surgery as the only surgical option.

1.4. Possibilities of prevention

Despite the fact that case numbers seem to have stagnated during the previous years, considering the exceptionally high mortality rate, primary and secondary prevention should gain more attention and emphasis, with the prior goal to fight this serious public health issue.

Primary prevention focuses on the avoidance of carcinogens, in the case of head and neck tumors it means the moderation of alcohol consumption and quitting smoking.

Secondary prevention (screening) aims at diagnosing the disease at an early, premorbid stage. At the borderline of primary and secondary prevention, molecular and predictive epidemiology plays a crucial role in tumor prevention. Predictive and molecular epidemiology is also at the borderline of primary and secondary prevention, with the help of early biomarkers as sensitive biological indicators it can detect any step in the exposition→disease

process prior to diagnostic markers of the definitive disease showing positivity.

Tertiary prevention includes patient rehabilitation and the prevention of complications, metastasis formation and recidivation. Methods include follow-up care and rehabilitation.

2. Theoretical background

2.1. Carcinogenesis

Carcinogenesis is a process including several stages, requiring genetic and epigenetic modifications within normal cells, triggered by various environmental effects, to turn into cancer cells. Based on recent research, the so-called cancer-field theory has been brought to life and has become widely accepted as a schematic description of the mechanism of the development of head and neck tumors.

2.2. Tumor markers

Tumor markers are biomarkers produced by the body the presence of which or changes in their concentration indicate the presence of a tumor.

Tumor markers can be divided into two main groups:

• Primary markers. They are molecules produced by tumor cells, changes in their quantity can be detected from blood or from a tissue sample taken from the tumor. Regarding their structure, they can be quite different: proteins, small molecular weight hormons, nucleic acids etc.

• Secondary markers. They are molecules produced by specific tumor growth mechanisms or responses of the organism. In theory, any measurable laboratory parameter can be regarded as a secondary marker. These parameters are not at all tumor specific, they only indicate a tumor being present, the body being affected.

2.3. Salivary diagnostics

The examination of proteins, protein fragments and other pathological biomarkers found in saliva significantly contrinutes to the development of modern diagnostics. Several researches are currently under way are aimed at identifying and detecting disease-specific proteins and biomarkers, since these are easily accessible, noninvasive, non-time-consuming non-expensive procedures that can be produced in large quantitites.

3. Research aims:

1. Our study aimed at investigating whether proteins can be detected in human saliva that can be considered as potential tumors-pecific biomarkers in head and neck tumors.
2. To what extent are these identified proteins specific to head and neck tumors?
3. Can proteins identified as potential tumor markers be found in tumorous and/or sorrounding tissue
4. To what extent can examination of the presence of these proteins in tissues characterise changes in the tumor’s microenvironment - tumorgenesis?
5. A further aim of our study was to provide a reliable examination protocol that is fast to carry out and relatively simple, which could later provide the basis for a study on a larger patient population.

4. Analytical Methods

4.1. Sampling

Saliva samples were collected after participants were asked to rinse their mouths with cold tap water, without stimulation between 8 am. and 10 pm. according to a widely accepted and applied protocol. Samples were collected from both the tumor group and the control group. Participants were asked to rinse their mouths three times with tap water prior to the

collection of samples; saliva samples were taken with 5ml syringes from the buccal fold in the non-stimulated oral cavity and were subsequently cooled on ice in Eppendorf tubes (Eppendorf Austria GmbH, Vienna, Austria). Thereafter, samples were centrifuged at 2500/min rpm for 12 minutes at 4°C. The supernatants of the saliva samples were subsequently stored frozen at -80°C until further tests.

4.2. Electrophoresis

The solutions available for the identification of proteins are most often not pure, they do not only contain one type of protein. These proteins have to be separated. Methods using electrophoresis have become the most popular separation methods. Their main principle is that in an electric field created within an aqueous environment, charged molecules drift towards the oppositely charged electrode. If the direction or speed of their movement is different, the molecules can be differentiated form each other. The speed of their movement depends on the charging of the molecule, its weight, the friction coefficient and the electric space.

In the case of proteins, the best resolution is provided by SDS-PAGE where proteins are denatured in the presence of sodium-dodecil-sulphate (SDS), thus they can be separated based on their weight alone. Calibrating with proteins of known molecular weight it becomes possible to determine the molecular weight of our protein.

4.3. Mass spectometry analysis

Mass spectrometry (MS), is a highly efficient analytical method based on the examination of weight/charging (m/z) of ions formed out of organic and non-organic components. The machine creates ions from the particles of the material analysed and then its analytical system separates these on the basis of their relative weight and the charge quotient.

4.3.1. Structure and functioning of the mass spectrometer

Components of the mass spectrometer: sample preparation-upload system, ion source, analyser, detector and the supporting computer data processing and directing system.

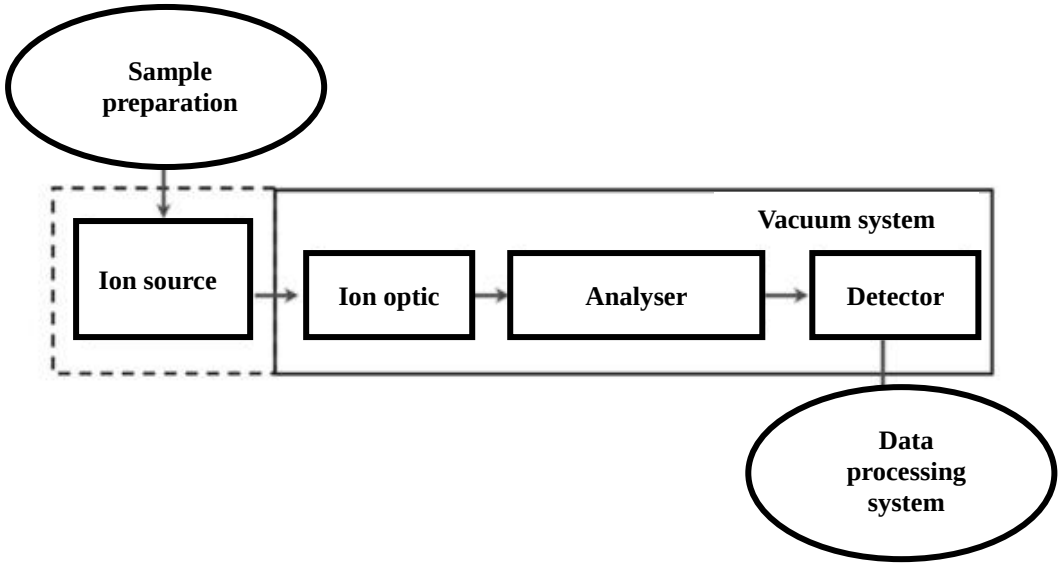

Figure 1.: Schematic outline of the operation of the mass spectometer

4.3.2. Preparation of samples

Theoretically, mass spectometry makes it possible to analyse any multi-component system of any consistency, however, this is largely influenced by the method of sample administration used and the method of ionisation. Volatile substances (e.g.: gases, easily evaporating substances) can be directly fed into the source where ionisation takes place.

4.3.3. Ion sources

For measurements to be carried out, the examined particles need to be ionised. The method of ionisation chosen depends on the molecule to be analysed and the sorrounding matrix. As there is no universally applicable ionising technique, the speed and simplicity of changing the various ion sources play a pivotal role in the improvement of machines.

4.3.4. Analyser

Separation of ions generated in the analyser is carried out on the basis of their weight and the charge quotient. The smaller the weight of the ion and the higher the charge, the

higher the speed it can acquire at a given accelerating voltage. A qualitative analysis can be performed, the machine is able to carry out measurements requiring high throughput (high throughput type), as it can do 6500 measurements daily in an automated mode.

4.3.5. Detector

The clusters of ions drifting inside the mass spectrometer practically mean an electric current moving from its source towards the detector. Detectors of the machines sense the arriving incredibly small ion current (10–10–10–15 A) and generate an analogous electric signal proportional to their value.

4.3.6. Fragmentation and tandem mass spectometry

If a relatively low energy is used for the ionisation of our substance molecules, these molecules remain whole and a so-called molecular peak [M+, M–] can be acquired, that is characteristic of their weight and charge. In most cases however, considerably more information can be gained, or we can find better solutions for special problems (e.g. separation of several substances, sequencing) if particles are crushed or fragmented with adding kinetic energy in a precisely regulated way. This process can occur during the ionisation process or shortly after (in source and post source decay, ISD, PSD), or also inside the analyser through a collision with an inert gas (collision induced dissociation, CID; and electron transfer dissociation, ETD).

4.3.7. The mass spectrum

Mass spectrum is the graph-like representation of the weight/charge ratio and ion intensity. The highest peak is the base peak which all other components are related to. Absolute ion mass is proportional to the amount of substance mass present in the sample, relative ion intensity of fragments is characteristic of the type of molecule and the method of measurement applied.

4.3.8. Identification of proteins - PMF (Peptide Mass Fingerprinting)

Investigations based on searching against databases for the products of proteolitic cleavage is based on the fact that peptides, derived via enzymatic digestion of proteins of a given specificity or via a chemical way, have a weight charateristic of their sequence. This means that a protein with a certain sequence yields a list of weights when digested, that can subsequently be compared with weight lists of peptides resulting form the theoretical digestion of proteins in the database upon a search. Such database search programmes include: Mascot, SwissProt, NCBInr. Search against the database uses the peptides' MS/MS spectrum on the basis of which, potential amino acid sequences are determined and then compared to the sequences of proteins in the database.

4.4. Imaging mass spectometry (MALDI-IMAGING)

At present, the most promising method in peptide and biomarker diagnostics is an imaging technique (MALDI Imaging) using MALDI TOF/TOF mass spectometry, during which 10-20 μm tissue specimens and matrices are mounted dry onto special slides. Subsequently, several thousands of mass spectra are acquired according to a a predetermined system and laser intensity. These are then visualised by the software in the form of an image according to the intensities mathching the m/z values. A considerable advantage of this method is that it can be performed paralel with other imaging techniques (in vivo fluorescent, NMR, MRI, CT, FT-IR), thereby, they can yield such novel scientific and diagnostic findings that may revolutionise the diagnostics of clinical methodology. MALDI imaging provides the possibility to examine proteins directly, in situ on tissue surfaces or on thin tissue samples while keeping the integrity of the tissue structure. Consequently, the images produced are very similar to the original tissue samples and also show the spatial arrangement of molecular components. Thus, it is not only the difference between healthy and unhealthy tissues that can

be visualised, but it also makes possible to notice such early stages of malignant transformation that could not yet be detected histologically.

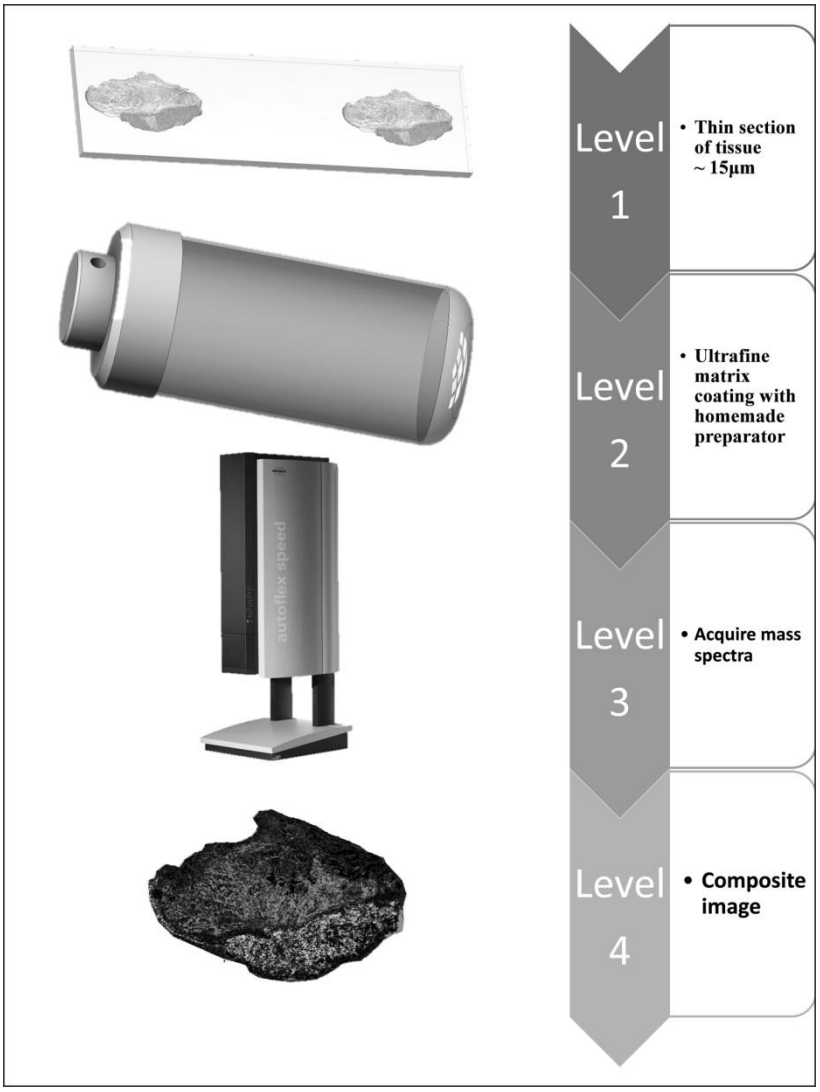

Figure 2. The process of imaging mass spectrometry

4.5. Immunohistochemistry, confocal microscopy

Following staining, the morphological features of cells can be examined with traditional imaging techniques, but via a specific marking with antibodies it is also possbile to obtain more precise molecular information. With the help of specific poli- and monoclonal antibodies generated against intracellular and surface proteins of cells, immunohistochemical methods using enzyme or covalent-bound fluorescent signalling we can make microscopic

observations of components of cellular proteins. Confocal microscopy is able to examine samples with fluorescent staining. The images acquired are of better resolution than those a traditional fluorescent microscope would give, and a confocal microscope is also able to carry out "optic slicing", i.e. it can provide an image of only one single slice from the sample. The digital image acquired can be processed and analysed by a computer. Accumulating images of several slides can allow for the examination of the spacial arrangement of the fluorophor.

5. Results

Our research had two main phases. The initial phase included the proteomic analysis of saliva samples obtained from patients with the aim to identify protein biomarkers. In the second phase, samples derived from histological samples taken from patients were examined with mass spectometry in order to determine the local distribution and expression of proteins previously identified as potential biomarkers. The present thesis follows the same structure, thus, the results section discusses the results of two multimodal studies on two patient populations with different participant numbers.

5.1. Identification of protein biomarkers from saliva samples

5.1.1. Patients

A total of 25 consenting patients with head and neck cancer participated in our study (10 females, 15 males); mean age was 56,48 years (the youngest was 22, the oldest was 78 years old). Head and neck cancer patients were compared with a control group composed of 25 healthy volunteers (15 smokers, 10 non-smokers) matched for age and sex. In the tumor group 19 patients had malignant, 6 patients had benign head and neck tumors. All patients diagnosed with sqamous cell carcinoma were heavy smokers (at least 1 pack/day). None of the patients had undergone treatment prior to saliva sample collection.

5.1.2. SDS-PAGE

Prepared samples were run on SDS-poliacrylamid gel, to separate proteins according to their size. Electrophoretogramm clearly presents the difference between the composition of saliva samples of the control and tumor groups.

5.1.3. Mass spectometry analysis

Subsequent to electrophoresis, gels were scanned and analysed visually. The extra sections appearing on the patents' samples were then excised with a scalple.

Mass spectometry analyses were performed using Autoflex II TOF/TOF types (Bruker Daltonics, Bremen, Germany). For MALDI TOF „peptid mass fingerprint (PMF)", LIFT mode for PSD (post source decay) and CID (collision induced decay) fragmentation was applied in an automated mode assissted by FlexControl 2.4 computer programme. During measurements, mass spectra were acquired between 800 and 5000 m/z, each spectrum was produced by accumulating data from 500 consecutive laser shots.

For the PMF identification of proteins MSDB (Swiss-Prot) and NCBInr databases and then the MASCOT database (MASCOT Server 2.2 search engine, MatrixScience Ltd., London, UK) search engine and Bruker BioTools 3.0 software (BrukerDaltonics, Bremen, Germany) were used. Proteins, the presence of which could be detected during disease manifestation and which could have potential diagnostic value, were identified via MALDI-TOF/TOF mass spectrometry. During the identification, the primary mass spectrum was recorded and thereafter, peptides of higher intensity or those of greater diagnostic significance were further dissected with PSD,- or CID fragmentation. This allowed us to identify the exact primary structure of peptides and structural modifications.

Well-known proteins with a general function were found in larger amounts in the samples taken from cancer patients and healthy subjects as well, such as amylase, keratines and actin. Additionally, increased levels of annexin 1, zinc finger protein 28, regulator G-

protein 3, indoleamine 2,3-dioxygenase, OFD1 protein and CEP290 proteins were found in most of the saliva samples from malignant cancer patients.

5.2. Local expression of proteins identified as biomarkers with imaging mass spectometry

In our study, we identified several proteins that had been identified as biomarkers earlier, the appearance of which was limited to a neoplastic or stromal environment. In this thesis I would like to highlight only two, the presence of which in neoplasms of epithelial origin has continued to raise questions to date. These two proteins are S100A8 and A9, both of which belong to the group of calcium-binding S100 proteins.

5.2.1. Patients

In this phase of our study our patient group consisted of 32 patients with head and neck cancer (6 females, 26 males), mean age was 60,44 years (age range:43 - 81 years) (appendix: Table 4.). Saliva samples were collected from all patients as described above. Subsequently, all patients underwent so-called direct laryngoscopy according to the examination guideline for head and neck patients.

During the examinations, samples were taken from the suspicious laesions and also from the sorrounding stromal and also distant, macroscopically healthy-looking tissues as well. Histologically, all samples proved to be well-differentiated squamous cell carcinomas. According to in situ hybridisation measurements all samples were HPV negative.

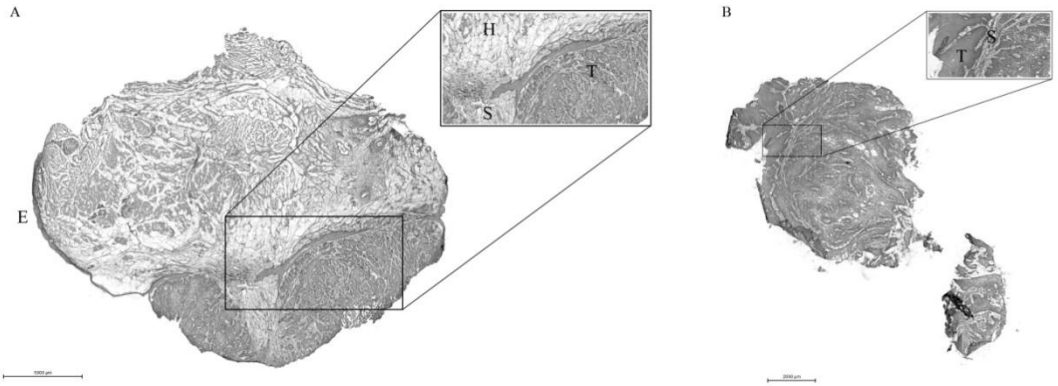

Figure 3.: Tissue sample with standard haematoxylin-eosin staining. Both samples show well-differentiated sqamous cell carcinoma with low mitotic activity, minimal atypicality and minimal signs of keratinisation.

5.2.2. Imaging mass spectometry

The CMC embedded samples from the selected head and neck cancer patients were prepared for MALDI IMS: regions that proved histologically non-maligant were used as controls. In this study we investigated the local distribution of S100A8 and S100A9 with the use of proteins with the help of MALDI TOF / TOF mass spectometry imaging. It was apparent that the overexpression of calgranulins is localised onto malignant and neostromal regions, it could not be observed in non-malignant, healthy tissues. Significant differences were found between malignant and healthy regions. It was to our surprise to find the presence of calgranulin on non-maligant epithelial surfaces, where a mild hyperplasia was noticeable but without any significant inflammation or invasivity.

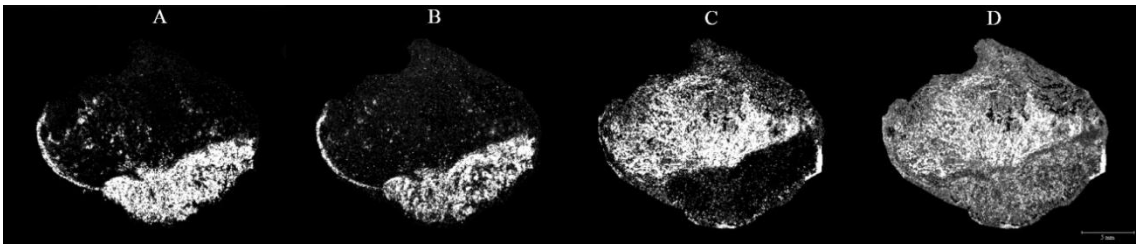

Figure 4.: Distribution of investigated proteins with imaging MS on B2 histological samples. S100A8 (**A**), S100A9 (**B**), haemoglobin alfa chain (**C**). **D:** composite image of the distribution of the three proteins

6. Discussion

The past few years have seen a significant development in proteomics, we are now able to identify and characterise proteins extracted from cells, tissues or biological fluids and also to determine their relative quantities. The significance of proteomics lies in that it allows for the direct protein-level analysis of signal transmission, regulation, enzyme activity and structural features that are coded at a genome-level and carried out at the protein level. The early diagnosis and treatment of primary or recidivating tumors is of utmost importance for the improvement of the still low survival rate of head and neck sqamous cell cancers.

In the first phase of our study, we identified proteins from human saliva samples that could be considered as potential molecular biomarkers for head and neck sqamous cell carcinoma.

During the second phase, we used MALDI for the molecular analysis of S100A8 and A9 in tissue samples and human saliva. Our results showed that S100A8 and A9 are present in the saliva as potential biomarkers in HNSCC. Furthermore, the overexpression of proteins can be observed in malignant tissues and the sorrounding stromal region but it cannot be detected in healthy tissue regions.

7. Summary of results

1. The method we applied allowed us to identify several low-molecular-weight peptides from human saliva which are in close association with head and neck sqamous cell carcinoma. Despite the fact that their exact role in carcinogenesis still remains to be more precisely identified, they are likely to be considered as biomarkers of early carcinogenesis. Although these peptides had been isolated from sera or malignant tissues earlier, our research group was the first to identify these as biomarkers.

2. Our results show, that as potential biomarkers, S100A8 and A9 are present in human saliva in case of head and neck sqamous cell carcinoma.

3. Our studies revealed a significant elevation in the local expression of S100A8 and S100A9 in histological samples. The overexpression of proteins can be observed in malignant tissues and the sorrounding stromal regions but cannot be detected in healthy tissue regions.

4. The upregulation of calgranulins in malignant and non-malignant but hyperplastic epithelium, that have been proven to play a role in cell-cycle regulation, suggests that S100A8 and S100A9 peptides can function as potential biomarkers in quite early stages of tumorigenesis by being indicators of changes in the microenvironment of tumors.

5. Our study protocol proved to be a well-functioning procedure that is standardisable and reproducible. The firm identification of biomarkers may foreshadow the possibility of screening examinations.

8. Acknowledgements

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9. Scientific publications

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