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Oral squamous cell cancer (OSCC) represents a major public health burden world-wide (*Ferlay J et al. Int J Cancer 2015; 136:E359-E386.*). OSCC mortality rate exhibited a striking 5-fold elevation in Hungary between the 1960-ies and the new millennium (*Suba Z et al. Fogorv Sz. 2009; 102:63-68.*). Presently, Hungarian males and females occupy the first places in cancer statistics in Europe with respect to both age-standardized incidence and mortality rates (*Ferlay J et al. Eur J Cancer. 2013; 49:1374-1403.*). Most OSCC is being diagnosed in advanced stages and long-term survival results don't exceed 50% despite recent advances in surgical methods, radiotherapy, and chemotherapy (*Kademani D et al. J Oral Maxillofac Surg 2005; 63:1599-1605.*). Early detection followed by appropriate treatment can increase the recovery rate up to 80% (*Kessler P et al Strahlenther Onkol 2007; 183:184-189.*). Timely diagnosis of OSCC and precancerous oral mucosal lesions, i.e. oral leukoplakia (OLK), and oral lichen planus (OLP) is an attractive strategy to decrease morbidity and mortality, to enhance survival rates and to improve quality of life of patients.

Saliva is a complex, informative body-fluid harboring biological molecules from multiple sources with the capacity of representing different diseases including OSCC and also general health. Attempts to find salivary biomarkers to identify OLK, OLP and OSCC by advanced molecular ('omics') methods aiming at salivary genomic, transcriptomic, proteomic, and metabolomic markers ('salivomics') resulted in the discovery of several candidate proteins, mRNAs, and miRNAs. However, the role of these molecules as diagnostic or prognostic biomarkers is often controversial and characteristic marker patterns exhibit a broad variation between patients of different ethnic groups (*see SahebJamee M et al. Med Oral Patol Oral Cir Bucal 2008; 13:E292-E295., Wu JY et al. Oral Oncol 2010; 46:226-231., Panta P, Venna VR. Anal Cell Pathol 2014; Article ID 450629., Khan RS et al. Proteomes 2016; 4, 41; doi:10.3390/proteomes4040041., Radhika T et al. J Oral Biol Craniofacial Res 2016; 6:S51-S54., for review*). The integration of the data from omics technologies, using saliva as a non-invasive and low-cost diagnostic tool, may contribute to early diagnosis of OSCC, to a better understanding of the pathobiology of OSCC and oral premalignant lesions, and to the development of targeted treatment approaches.

The aim of this research project was to investigate OSCC-related salivary transcriptome, proteome and metabolome patterns, so as to develop and to validate a candidate biomarker panel by obtaining samples from a Hungarian nation-wide multicentric patient cohort. The strategy and design af the investigations followed a step-by-step approach. First we applied both targeted and high-throughput molecular methods to identify potential biomarkers in a 'test'

sample set obtained from a pilot cohort of patients and controls of the Debrecen center. Next, potentially useful markers were validated by 'gold standard' methods on a 'reference' sample set obtained from different patients of the same pilot cohort. Results were then analysed, the experimental methods were optimized and the most robust potential biomarkers were investigated on a 'validation' sample set obtained from the nation-wide multicentric patient cohort referred from the Faculty of Dentitry of the University of Debrecen, Debrecen, the Faculty of Dentistry of the University of Szeged, Szeged, the Department of Dentistry, Oral and Maxillofacial Surgery, of the Medical School, of the University of Pécs, Pécs, and the Faculty of Dentistry of the Semmelweis University, Budapest, (for further use: Debrecen, Szeged, Pécs and Budapest centers, respectively) representing different geographic regions of the country. The impact of "omics" signatures was investigated by comparing newly identified biomarkers with conventional demographic, clinical and pathologic markers of OSCC.

Molecular targets were selected based on a comprehensive review of available results from the scientific literature. A special emphasis was given to the observations of the 'Wong group' which was the first to report on potentially useful salivary mRNA and protein biomarkers in patients with OSCC and to publish salivomics results of patients with OSCC from Serbia, a population similar to Hungarian patients with respect to socio-economic status, oral and dietary habits and cancer statistics (*St John MA et al. Arch Otolaryngol Head Neck Surg 2004; 130:929-935., Brinkmann O et al. Oral Oncol 2011; 47:51-55.*). Since immune-inflammatory molecules were repeatedly reported among the best performing OSCC salivary biomarkers, we performed a comprehensive literature survey investigating the possibility that non-neoplastic oral inflammatory lesions may represent an alternative source of salivary immune-inflammatory biomarkers. Based on the literature survey and on our own previous results, with the support of this grant, we have published a review article confirming the above hypothesis (***Márton IJ, Kiss C. Overlapping protective and destructive regulatory pathways in apical periodontitis. J Endod 2014; 40:155-163.***). Taken the characteristic age distribution of patients with OSCC and the poor oral hygiene conditions of the elderly Hungarian population resulting in the presence of oral inflammatory conditions in the majority of index individuals, we have decided on using two types of control groups in our project, a group of age- and gender-matched controls to patients with OSCC, and a young control group with uncompromised oral health conditions.

In addition to the salivomics investigations, histological blocks of patients from the Debrecen center were subjected to immunohistochemical (IHC) analysis by an independent pathologist so as to check the relationship of salivary biomarkers with tissue-derived biomarkers identified

in OSCC samples. Moreover, with the support of this grant we have developed and published an instrument for checking self-assessed oral health-related quality-of-life of patients (***Jenei Á., Sándor J, Hegedűs C, Bágyi K, Nagy L, Kiss C, Szabó G, Márton I. Oral health-related quality of life after prosthetic rehabilitation: a longitudinal study with the OHIP questionnaire. Health Qual. Life Outcomes 13 (99), 1-7., 2015.***). We also have submitted a review article summarizing the significance of the most frequent side-effect of cancer management, which is oral mucositis. This article has been accepted for publication (***Nemes J, Jenei Á, Márton I: A gyermekkori malignus kórképek kemoterápiájának leggyakoribb mellékhatása, az orális mucositis. Irodalmi áttekintés. Orvosi Hetilap (in press))***.

Recruitment figures and demographic and clinical characterization of the patients:

Between Januar 1, 2013 and September 30, 2017, 107 saliva samples of patients with OSCC were collected together with a complete pathological and clinical data set from the Debrecen, Szeged, Pécs, and Budapest centers. Saliva samples of patients with OLP (38) and OLK (10) were underrepresented. The explanation of the less-than-expected premalignant sample size can only be hypothesized: OLP and OLK lesions are usually painless and are not easy to be discovered by self-assessment. Unfortunately, oral haelth consciousness including participation in regular dental check-up examinations are much less frequent than desirable among the Hungarian population. Therfore, OLP and OLK might have remained underdiagnosed both in the local pilot and the nation-wide cohort. Sampling and investigations of the samples were performed after written informed consent of enrolled patients and controls based on the permission from the national review board, the Scientific Research Ethics Committee ('TUKEB').

Among investigated patients there were 71% males and 29% females. The age ranged between 41 and 87 years. According to these figures, the onset of the OSCC inclines definitely towards the younger ages. The most common localization of the tumors was the tongue followed by the floor of the mouth, the gingiva and root of the tongue. In nineteen cases the tumor appeared in two, while in one cases in three different sites of the oral cavity at the same time, emphasizing the late discovery of some OSCC cases. The three most common registered TNM groups were the followings: 22,4 % T2N0M0, 15 % T1N0M0, and 9,3% of the tumors belonged to the T4N0M0 group. Regarding the stage distribution, 35,5% of the OSCC patients belonged to stage IV, 24,3 % of the patients to stage II and 19,6%-19,6% belonged to the stage I and III. One percent was not available. The histological grade was 2 (moderately differentiated) in 55,1%, 1 (well differentiated) in 21,5% and 3 (poorly differentiated) in 16,8 % of the cases.

Data in 6.6 % of the patients are not available. Demographic and clinical data of patients have been analized by biostatistical methods and will be published shortly.

Proteomics and metabolomics investigations:

In the pilot cohort of patients with OSCC form the Debrecen center we investigated 14 proteins which were previously reported as significantly elevated in saliva of patients with OSCC. The 'test' set contained 29 patients with OSCC, 25 age-matched controls, and 8 young controls. The 'reference' set contained 26 patients with OSCC, 12 age-matched controls, and 7 young controls. In case of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and VEGF a Luminex-based multiplex kit was utilized and the salivary concentrations were determined. In case of catalase, profilin-1, S100A9, CD59, galactin-3-bindig protein, CD44, thioredoxin and keratin-19, selected reaction monitoring (SRM)-based targeted proteomic method was developed and the relative amount of the proteins was determined in the saliva of patients with OSCC and controls. After several rounds of optimization and using stable isotope-containing peptides, we developed an SRM-based method for rapid salivary protein detection. The validation of the selected potential biomarkers by ELISA revealed salivary protein S100A9 and IL-6 as useful protein biomarkers for OSCC detection improving the diagnostic accuracy for OSCC in the Hungarian population. In the case of these two salivary proteins we have observed significant differences both between the patient group vs. age-matched controls and the patient group vs. young controls. The distribution of S100A9 and IL-6 concentration was similar in the age-matched and young control groups. Results of this study were published (***Csősz É, Lábiscsák P, Kalló G, Márkus B, Emri M, Szabó A, Tar I, Tőzsér J, Kiss C, Márton I. Proteomics investigation of OSCC-specific salivary biomarkers in a Hungarian population highlights the importance of identification of population-tailored biomarkers. PLoS One 2017; 12(5) eo177282.***).

In a second set of experiments, we have used samples of 40 patients of the same preliminary cohort of the Debrecen center. In a search for novel salivary OSCC protein and metabolic biomarkers three randomly selected samples ('test' set) from patients with OSCC and matched controls, respectively, were subjected to shotgun proteomics analysis. We performed LC/MS/MS analysis using ultra-Performance Liquid Chromatography (UPLC) coupled to a linear ion trap-Orbitrap hybrid tandem mass spectrometer. More than 500 proteins were identified from salivary samples and 2 proteins, the cytochrome c and mucin-7 were only present in the control samples and 6 proteins, the complement factor H (CFH) and C5 (C5), corticosteroid binding globulin (SERPINA6), heparin cofactor 2 (SERPIND1), apolipoprotein E (APOE) and serum paraoxonase/arylesterase 1 (PON1), were only present in the OSCC samples. The functional examination and the analysis of the network of proteins differentially

expressed in OSCC revealed extensive cancer-related changes. The levels of apolipoproteins, components of the complement system, proteinases, proteinase inhibitors, components of the coagulation cascade were upregulated indicating a change in the lipid metabolism and in the proteolysis most probably associated to the interrelated coagulation cascade-complement activation processes. In the same time the level of proteins having role in carbonhydrate metabolism and host defense was downregulated. Considering the proteins present only in OSCC based on our shotgun experiments, the data presented in the literature and the availability of antibodies, SERPIND1 and C5 were selected for further studies. In order to test the utility of potential biomarkers identified in Asia for a European population, resistin found to be a potential biomarker for OSCC in Taiwan, but not in our shotgun experiment, was also selected. The concentrations of C5, SERPIND1 and resistin were examined in the saliva of 37 patients with OSCC, age-matched and young controls using quantitative sandwich ELISA kits ('reference' set). In case of C5 the difference was significant but only when young controls and patients with OSCC or young controls and age-matched controls were compared indicating that the level of C5 was rather age- than OSCC dependent. In case of resistin and SERPIND1 no significant differences were found between the groups. This outcome highlights in one hand the importance of validation of the shotgun proteomics data to decrease the false positivity of biomarker identifications and on the other hand the importance of regional and population-tailored studies. These results were summarized and submitted for publication in 2017 and accepted for publication before the deadline of the final report of the project (***Csősz É, Márkus B, Darula Z, Medzihardszky KF, Nemes J, Tőzsér J, Kiss C, Márton I. Salivary proteome profiling of oral squamous cell carcinoma in a Hungarian population. FEBS Open Bio [in press].***).

Along with the proteomic changes characteristic for OSCC, saliva samples from patients with OLK and OLP were also examined. One- and two-dimensional electrophoresis was carried out and the differentially expressed bands were analyzed by mass spectrometry. With the support of the present grant, three randomly selected samples from each group of samples from patients with OSCC, OLK, OLP and controls, respectively, were sent for analysis to the OLink Proteomics in 2017 to determine the relative amount of proteins belonging to the 'Inflammation' (http://www.olink.com/products/inflammation/) and 'Oncology' panels (http://www.olink.com/products/oncology/). Results are under evaluation and soon will be submitted for publication.

Transcriptomics investigations:

We evaluated the performance of seven previously identified, potential mRNA biomarkers of

OSCC in 31 saliva samples of patients from the pilot cohort of the Debrecen center. Expression of the putative OSCC bio-markers (DUSP1, OAZ1, H3F3A, IL-1B, IL8, SAT and S100P), two biomarkers of inflammation (IL-6 and TNFα) and eight putative normalizing genes was quantified from each sample using real-time quantitative PCR. Salivary expression of IL-6 mRNA has not yet been quantitated in patients with OSCC. In contrast to previous studies, the expression pattern of the seven mRNA biomarkers was similar between OSCC patients and age-matched control patients in the Hungarian patient population. On the other hand, five of the seven mRNA biomarkers were present at significantly higher levels in saliva samples of OSCC patients when compared to young control patients. The best biomarker combination could distinguish only the OSCC vs. young control patients, but not the OSCC vs. age-matched control patients. In conclusion, the significant differences between our results and previous studies, and the clinical characteristics of the patients suggested that inflammatory processes in the oral cavity might have affected the performance of the seven putative salivary mRNA biomarkers. Lastly, since IL-6 mRNA was quantifiable in the majority of OSCC cases, but only in a few control samples, salivary IL-6 mRNA may be utilized as part of a biomarker combination to detect OSCC. Results of this study were published (***Horváth J, Szabó A, Tar I, Dezső B, Kiss C, Márton I, Scholtz B. Oral Health May Affect the Performance of mRNA-Based Saliva Biomarkers for Oral Squamous Cell Cancer. Pathol Oncol Res. 2017, 23, 1-10***).

In order to identify more sensitive and specific salivary RNA biomarkers of OSCC, in 2017 we investigated the miRNA expression pattern in the saliva of patients with OSCC. Instead of a global miRNA profiling, we focused our analysis on a limited number of miRNAs expected to be overexpressed in saliva samples of the cancer patients. These miRNAs were selected from several studies in the scientific literature that identified OSCC-specific, overexpressed miRNAs in the tumors, and fulfilled the following criteria for study design: identification of tumor-specific miRNAs by global analysis, on a sufficiently high patient number, followed by qPCR-based validation on an independent patient cohort. Based on these publications, we selected the following miRNAs for validation: hsa-miR-31-5p, hsa-miR-424-3p, hsa-miR-184, hsa-miR-191-5p and hsa-miR-345-5p. These miRNAs were identified as OSCC-specific miRNAs in at least two independent studies, with the exception of hsa-miR-191, which was identified in a Hungarian patient cohort. In addition, these miRNAs are not connected with the regulation of inflammatory processes, based on the scientific literature. SNORD104 and SNORD60 small RNAs were used as reference genes for the qPCR. In the initial experiments we utilized PCR preamplification followed by qPCR quantification, to ensure succesful quantification even from

the most dilute samples. Based on these experiments, the differences of the selected miRNAs were not statistically significant between the cancer and control patients ($p > 0.05$ Mann-Whitney U test). However, several miRNAs gave unexpectedly high signals in the preamplification+qPCR experimental setup, indicating the possibility of overamplification. Therefore, we repeated the measurements for miR-31 and miR-191, plus miR-21-3p, this time without pre-amplification. miR-21 was used as a kind of positive control – even though its role in inflammatory processes is well documented both in normal and tumor tissues, miR-21 was shown to be overexpressed in OSCC tumor tissue by several research groups. As expected, 10-30% of samples failed to give quantifiable results without preamplification. Comparison of the positive samples revealed that all three miRNAs were present in significantly higher quantities in the samples of cancer patients vs. control patients. This suggested that the preamplification step, indeed, overamplified the target sequences, distorting the results and minimizing the differences between the patient groups. Therefore, all miRNA qPCRs without preamplification will be repeated shortly with the help of the present grant support. Based on the ROC analysis, none of the miRNAs were able to differentiate reliably by themselves the cancer and control patients: AUC(miR-31)=0.77, AUC(miR-191)=0.75 and AUC(miR-21)=0.70. Combination of the three miRNAs, on the other hand, gave much better results (logistic regression analysis): AUC=0.81. Repeated experiments have been started and will be finished soon. Results will be analyzed and will be submitted for publication in this semester. The present project will be spelled out as grant support.

Histological investigations:

Oro-pharyngeal squamous cell carcinoma (OSCC) is the most common histotype of cancer in the oral cavity which invariably arises from malignant transformation of the dysplastic-precancerous mucosa lesion due to the presence of significant and long-lasting detrimental environmental factors (smoking and alcoholic abuses) or the cytopathic effects of high-risk type HPV agents that integrate into the epithelia's genome. Despite of the extensive research, yet only few OSCC-associated biomarkers are available that could be used innovatively for a cancer-screening from either blood or saliva samples, and/or to develop suitable strategies to establish a powerful targeting therapy. In an effort of such research projects, it is indispensible to take into account about the diversity of the cancer's biological behavior, its pleiotropic immune-editing capacities (that usually causes suppression), as a result of genetic heterogeneity due to random and multiple gene defects of transformed neoplastic squamous cells which ultimately acquire consecutive disturbances in cellular maturation and differentiation with versatile and aberrant protein-expression profiles and may produce dysregulations to promote

and facilitate tumour growth at the local and distant tissue sites. It is widely accepted that some of these features may also block the beneficial anti-cancer immune response against neoplastic cells mediated by the tumour infiltrating leukocytes (TIL) which in turn resulting in cancer progression.

In view of this concept depicted above our genomic and proteomic data obtained from saliva samples of patients with OSCC should be correlated with the corresponding cancer's morphology, the grade of differentiation, staging, the rate of cellular proliferation, the growth- and protein expression patterns of tumour cells in relation to the quantities and qualities of TIL detectable in the tissues. Therefore, investigated cases were evaluated for the the density of TIL, the characteristics of inflammatory cells' phenotypes present in the TIL, also, which may reflect the overall direction of cancer-associated immune-modulation.

Morphological and immunohistochemical analyses:

The slides of the retrieved tissue samples of the enrolled **(**56 No of**)** OSCC cases were microscopically reviewed again by an independent pathologist to reconfirm the original diagnosis including the histotype, grading, staging, together with the markers for the prognostic and predictive factors made earlier (e.g., Ki67 IHC for proliferation and the density of TIL, etc).

In addition, 41 representative formalin fixed and paraffin embedded tissue blocks of the 56 OSCC cases (that were treated in Debrecen) were used for IHC for other markers: CD163; CPM (carboxypeptidase M); CD3; CD4; CD8; CD20; IL-6; PD-L1; p16; p53; IMP3 (**I**nsulin-like growth factor II mRNA binding protein 3). The IHC reactions were made on deparaffinized tissue sections, after antigen retrievals under high pressure heat conditions (at pH6.0 or pH9.0). In turn, mouse or rabbit monoclonal primary antibodies for the above markers (from DAKO, Abcam, Biogenex, Novocastra) were applied followed by peroxidase-conjugated secondary immunoglobulin treatments using peroxidase-based detection kits (DAKO) and VIP or DAB chromogenic substrates (Vector Labs) as previously described in detail.

The stained sections were then digitalized using Panoramic MIDI digital slide scanner (3D-Histech-Zeiss, Budapest, Hungary) equipped with Hitachi (HV-F22CL) 3CCD progressive scan colour camera. Image analysis was performed by the HistoQuant application of Panoramic viewer software 1.15.2 (3D-Histotech) as described in detail earlier.

Our results showed that the peri- and the intratumoral density of tumour infiltrating leukocytes (TIL) primarily depended on the size of the cancer, the growth-pattern of the neoplastic cells, and the extent of host tissue destruction or tumour necrosis, and it positively correlated with the

tumour-grade, as well. Accordingly, those OSCC lesions exhibiting poor differentiation (grade 3) with high rate cellular proliferation and/or progressive infiltrative growth pattern with necroses or ulcer formation, appeared to harbour an increased number of TIL as compared to low grade and early (small) carcinomas - albeit the level of increase in inflammatory cells was found not significant for the entire cases. Based on the morphology and IHC finding, the TIL was essentially and predominantly composed of CD3+ immune T-cells and many CD163 positive macrophages, occasionally with the presence of few CD20+ B-lymphocytes, plasma cells, and/or eosinophils. In case of necrosis or ulcer, TIL cells were admixed with dense polymophonuclear leukocytes (PMNs) within fibrin-rich exudate, however, with low grade granulation tissue formation. Besides macrophages/histiocytes, the TIL's most abundant cellular components were the T-lymphocytes that comprise mainly of CD4+ helper T-cells and to a lesser extent CD8+ cytotoxic T-cells. The helper T-cell's predominance, however, did not show significant correlation neither with the tumour-size nor the tumour-grade or the anatomical site of the lesion. The ratio of tumour-associated cells for CD4:CD8 was calculated 2:1 in 73% of the cases (30/41), reflecting physiological (normal) homeostasis at tumour sites, which suggested that no powerful cytotoxic anti-tumour immune reaction likely developed in most of the cases. Only five cases were recorded (12.2%) where the ratio of the tumour-associated cytotoxic T-cells and helper T-cells in TIL proved to be 2:1 (indicating inversion) but no unambiguous microscopic signs for any tumour cell destruction could be noted.

In accordance with the morphological and immunohistochemical observations indicating attenuated anti-neoplastic immune responses, we made IHC staining for the detection of the presence of PD-L1 protein known to be involved in T-cell mediated anti-cancer immune-suppression. Indeed, in 68.3% of the cases (28/41) PD-L1 expression could be demonstrated in (CD4/CD25+) regulatory T-cells (Treg) and/or CPM-expressing M2 macrophages within the TIL. Furthermore, it was remarkable that in 9 cases the tumour cells also express PD-L1 amounting to 78% of cases (32/41) that show PD-L1 positive cancer-microenvironment that likely promotes an anti-tumour immune suppression.

Basically, similar findings were found with the pro-inflammatory cytokine, IL-6 which may also drive immune cells toward suppression: in 63% of cases (26/41) there were detectable expressions in TIL cells and in 16 cases tumour cells showed positive staining for the IL-6 amounting to 68.3% (28/41) that exhibited IL-6 positive tissue-environment at sites and around of neoplasia. The presence of tumor-infiltrating lymphocytes and macrophages, as well as the manifestation of IL-6 both by the tumor cells and the tumor-infiltrating immune cells supports

the rational of emerging immune-checpoint inhibitor therapy in OSCC and may offer an additional therapeutic target aiming at the IL-6-IL-6 receptor interaction.

In addition, IHC stainings for p16 and p53 were made, also. In 19.5% of the cases (8/41) p16 positive tumour cells were detected indicating HPV-association. On the other hand, 66% of the cases showed p53 positive cancer, probably reflecting OSCC with non-viral background. Both the p16+ and the p53+ cases did not show significant correlations with the qualities and quantities of TIL, PD-L1 and IL-6 expression patterns, respectively.

Finally, we made preliminary IHC stainings for the IMP3 (Insulin-like growth factor II mRNA binding protein 3) that is known to be an oncofetal protein which is never expressed in adult (differentiated) normal epithelial tissues. Remarkably, 65.8% of the OSCC cases (27/41) showed distinct and specific cytoplasmic and mebranous IMP3 expressions restricted to the squamous carcinoma cells, only, while leaving the normal cells negative. Based on these results demonstrating the high specificity and sensitivity for labelling squamos carcinoma cells but not the normal epithelia, IMP3 appeared to be one of the most useful potential biomarker for screening and identify OSCC lesions in early stages which might be carried out even from saliva samples in a non-invasive manner.

Results of the nation-wide, multicentric validation cohort:

Based on the results of investigations on the the pilot cohort, we decided to validate the performance of salivary IL-6 (protein) and IL-6 mRNA in 94 patients with OSCC. In the validation cohort we used only age-matched controls, since the distribution of IL-6 levels both at the protein and mRNA levels were similar among age-matched and young controls of the pilot cohorts. IL-6 protein concentration was determined by ELISA. Patients with OSCC had statistically significantly higher salivary IL-6 concentrations than controls. Using ROC analysis, salivary IL-6 protein concentration successfully identified patients with OSCC: AUC(IL-6 protein concentration)=0.80. However, there was an overlap between the distribution of salivary IL-6 protein concentration of the patient and the control group. Similar but more discriminative results were obtained by investigating salivary mRNA expression. The majority of saliva samples from cancer patients were positive for IL-6 mRNA, but there were some samples that gave no quantifiable results. To test the possibility of technical error, we repeated the reverse transcription and qPCR for the negative tumor samples (18 samples). At the same time, we repeated the measurements for 35 control samples (also tested negative previously). With the repeated qPCRs, 67 saliva samples of 70 saliva samples from the cancer patients tested positive for IL-6 mRNA, but the control samples tested negative even in the

repeated measurements. The IL-6 mRNA was present in significantly higher quantities in the saliva samples of the cancer patients, and based on the ROC analysis, the saliva IL-6 qPCR could excellently identify the OSCC patients: AUC(IL-6 mRNA)=0.97. Combination of miR-31, miR-191 and miR-21 with IL6 did not give better results (logistic regression analysis): AUC=0.92. Both salivary IL-6 protein concentration and mRNA levels correlated with the stage of the lesions (St I/II vs. III/IV). Manuscript is under preparation and will be soon submitted for publication.

Conclusions:

The grant support provided an excellent opportunity to investigate the differential expression of salivary markers among patients with OSCC and premalignant oral mucosa lesions vs. controls, for the first time in Hungary, using a complex set of molecular ('omics') methods. The presence of salivary IL-6 mRNA has not yet been quantitatively determined by other groups, leading to a significant novel observation of this project. The presence of salivary IL-6 mRNA, together with salivary IL-6 protein manifestation, was shown to be the single-most sensitive and specific salivary biomarker among Hungarian patients with OSCC. Salivary IL-6 expression both at the RNA and the protein level is biologically and clinically relevant, since parallel IHC results documented the manifestation of this immunoregulatory cytokine both by the tumor cells and the tumor-infiltrating immune cells, offering a rational and target for biological therapy. However, IL-6 is an inflammatory cytokine and its salivary expression (as much as the salivary expression of other immune-inflammatory biomatkers) may be compromised by the presence of inflammatory oral conditions coinciding with or independent of OSCC. In this context, another novelty of the present project is the introduction of young controls with uncompromised oral health in addition to age-matched controls. This experimental design has not yet been applied by other research groups. These observations underline the need for further search of salivary OSCC biomarkers which, in addition to IL-6 mRNA and protein, or independently may aid early, specific and sensitive detection of OSCC. With the help of the present grant support we have developed a complex set of optimized and validated advanced molecular methods suitable for achieving that goal.

Outstanding new results of this project have been published in (in extensor or electronically) five articles. Two additional manuscript have been accepted by respectful, peer-reviewed scientific journals, and four further manuscripts, summarizing the results of this project are about to be submitted within the next few weeks. Results were also disseminated as 24 lectures and eight poster presentations in the frame of Hungarian and international scientific

conferences. Based on the results of this project, one PhD student is about to prepare and to defend his PhD Thesis.

Summary:

Differential expression of salivary markers in patients with OSCC and controls was investigated by advanced molecular ‘omics’ methods. Among several protein and mRNA molecules, salivary IL-6 mRNA proved to be the best-performing biomarker to detect OSCC in the Hungarian population. Salivary IL-6 mRNA has not yet been quantitated by other groups. IHC results demonstrated the manifestation of IL-6 in tumor cells and tumor infiltrating immune cells supporting the clinical relevance of salivary IL-6. A further novelty of our project was the introduction of healthy young controls in addition to age-matched controls so as to differenciate between lesion-derived and inflammation-related markers. We have developed a complex set of optimized and validated advanced molecular methods suitable for further discovery of OSCC salivary biomarkers. Results supported by this grant have been disseminated in scientific publications and in scientific conference presentations.