Alterations in Oral Mucosal Microbiota of Patients with Oral Potentially Malignant Disorders

Ph.D. Thesis

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Publications Providing the Basis of the Thesis

I. **Decsi G**, Soki J, Pap B, Dobra G, Harmati M, Kormondi S, Pankotai T, Braunitzer G, Minarovits J, Sonkodi I, Urban E, Nemeth IB, Nagy K, Buzas K (2019) Chicken or the Egg: Microbial Alterations in Biopsy Samples of Patients with Oral Potentially Malignant Disorders. Pathol Oncol Res 25:1023-1033

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II. Harmati M, Gyukity-Sebestyen E, Dobra G, Terhes G, Urban E, **Decsi G**, Mimica-Dukić N, Lesjak M, Simin N, Pap B, Nemeth IB, Buzas K (2017) Binary mixture of Satureja hortensis and Origanum vulgare subsp. hirtum essential oils: in vivo therapeutic efficiency against Helicobacter pylori infection. Helicobacter 22(2)

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III. Hettmann A, Demcsák A, **Decsi G**, Bach Á, Pálinkó D, Rovó L, Nagy K, Takács M, Minarovits J (2016) Infectious Agents Associated with Head and Neck Carcinomas. Adv Exp Med Biol 897:63-80

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Further publications related to the topic of the thesis

1. Sonkodi I, Boda K, **Decsi G**, Buzás K, Nagy K. (2018) A clinicopathological retrospective epidemiological analysis of benign tumors and tumor-like lesions in the oral and maxillofacial region, diagnosed at the University of Szeged, Department of Oral Medicine (1960-2014). Orv Hetil 159:1516-1524.

2. Hettmann A, Demcsák A, Bach Á, **Decsi G**, Dencs Á, Pálinkó D, Rovó L, Terhes G, Urbán E, Buzás K, Nagy K, Takács M, Minarovits J. (2018) Prevalence and genotypes of human papillomavirus in saliva and tumor samples of head and neck cancer patients in Hungary. Infect Genet Evol 59:99-106.

3. Zsedenyi A, Farkas B, Abdelrasoul GN, Romano I, Gyukity-Sebestyen E, Nagy K, Harmati M, Dobra G, Kormondi S, **Decsi G**, Nemeth IB, Diaspro A, Brandi F, Beke S, Buzas K. (2017) Gold nanoparticle-filled biodegradable photopolymer scaffolds induced muscle remodeling: in vitro and in vivo findings. Mater Sci Eng C Mater Biol Appl 72:625-630.

4. Harmati M, Tarnai Z, **Decsi G**, Kormondi S, Szegletes Z, Janovak L, Dekany I, Saydam O, Gyukity-Sebestyen E, Dobra G, Nagy I, Nagy K, Buzas K.(2017) Stressors alter intercellular communication and exosome profile of nasopharyngeal carcinoma cells. J Oral Pathol Med 46:259-266.

5. Hettmann A, Demcsák A, Bach Á, **Decsi G**, Dencs Á, Pálinkó D, Rovó L, Nagy K, Minarovits J, Takács M. (2016) Detection and Phylogenetic Analysis of Torque Teno Virus in Salivary and Tumor Biopsy Samples from Head and Neck Carcinoma Patients. Intervirology 59:123-129.

6. Palmer SC, Ruospo M, Wong G, Craig JC, Petruzzi M, De Benedittis M, Ford P, Johnson DW, Tonelli M, Natale P, Saglimbene V, Pellegrini F, Celia E, Gelfman R, Leal MR, Torok M, Stroumza P, Bednarek-Skublewska A, Dulawa J, Frantzen L, Ferrari JN, Del Castillo D, Bernat AG, Hegbrant J, Wollheim C, Gargano L, Bots CP, Strippoli GF; ORAL-D Study Investigators. (2015) Dental Health and Mortality in People With End-Stage Kidney Disease Treated With Hemodialysis: A Multinational Cohort Study. Am J Kidney Dis 66:666-76.

7. Niller HH, Tarnai Z, **Decsi G**, Zsedényi A, Bánáti F, Minarovits J. (2014) Role of epigenetics in EBV regulation and pathogenesis. Future Microbiol 9:747-56.

8. Strippoli GF, Palmer SC, Ruospo M, Natale P, Saglimbene V, Craig JC, Pellegrini F, Petruzzi M, De Benedittis M, Ford P, Johnson DW, Celia E, Gelfman R, Leal MR, Torok M, Stroumza P, Bednarek-Skublewska A, Dulawa J, Frantzen L, Ferrari JN, del Castillo D, Hegbrant J, Wollheim C, Gargano L; ORAL-D Investigators. (2013) Oral disease in adults treated with hemodialysis: prevalence, predictors, and association with mortality and adverse cardiovascular events: the rationale and design of the ORAL Diseases in hemodialysis (ORAL-D) study, a prospective, multinational, longitudinal, observational, cohort study. BMC Nephrol 14:90.

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List of Abbreviations

CFU – colony forming unit

DNA – deoxyribonucleic acid

EBV – Epstein-Barr virus

HPV – human papilloma virus

LP – lichen planus

MALDI-TOF MS – matrix-assisted laser desorption ionization-time of flight mass spectrometry

OLP – oral lichen planus

OPMD – oral potentially malignant disorders

OSCC – oral squamous cell carcinoma

PVL – proliferative verrucous leukoplakia

qPCR – quantitative polymerase chain reaction

TTV – torque teno virus

1 Introduction

Leukoplakia and oral lichen planus (OLP) are the most frequent OPMDs in our clinical practice at the Department of Oral Medicine, Faculty of Dentistry, University of Szeged. Both disorders appear as whitish coloured hyperkeratosis, similarly to some other OPMDs. From the point of view of clinical differential diagnostics, this group of OPMDs belongs to a „Potentially malignant and malignant" subgroup (leukoplakias, submucosal fibrosis, lichen planus, lupus erythematosus, epidermoid carcinoma, verrucous carcinoma) of so called „White lesions". The group of „White lesions" also includes additional subgroups, such as the „Congenital", „Aquired infectious and non-infectios", and „Other" subgroups containing diseases with different etiologies. The possible causes of the whitish colour of „White lesions" include hyperkeratosis, pseudomembranous infection or inflammation. Due to this heterogeneity of diseases with the same clinical feature, clinical diagnostics of white lesions could be very difficult. Thus, quite frequently, pathological, immunological or microbiological support is needed to set up a proper diagnosis, prognosis and treatment plan.

Leukoplakia is defined as ″A white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer″. Leukoplakia is six times more common among smokers than among non-smokers. These lesions are divided into homogenous (simplex) and non-homogenous types. The non-homogenous types based on the cellular variability are verrucous leukoplakia, nodular leukoplakia and erythroleukoplakia. Proliferative verrucous leukoplakia (PVL) is a subtype of verrucous leukoplakia that shows resistance to treatment and a high rate of malignant transformation. It is more frequent among elderly women, in many cases without smoking and consumption of alcohol in the anamnesis. Distinct histopathological changes, like hyperkeratosis with or without dysplasia, may accompany the transition of PVL to verrucous hyperplasia and verrucous carcinoma. There are conflicting results regarding the association of leukoplakia and human papilloma virus (HPV) infection. The role of torque teno virus (TTV), Epstein-Barr virus (EBV) and *Candida albicans* in leukoplakia development and carcinogenesis remains to be clarified, too. Additionally, it was suggested that specific bacterial infections like *Helicobacter pylori*, or the intracellular *Mycoplasma salivarium* could also be involved in this process. As to the putative role of bacterial infection in oral carcinogenesis, one may speculate that it could be a process analogous to *Helicobacter pylori* infection of stomach which predisposes to the development

of gastric cancer. Thus, an *in vivo* murine model of *Helicobacter pylori* induced mucosal lesions could potentially mimic the lesions caused by other bacteria or bacterial communities on oral mucosal surfaces. As a matter of fact, a disbalance in the oral microbial flora may play a role in the development of leukoplakia and carcinogenesis, as suggested by the association of periodontal inflammation with leukoplakia and a changing bacterial flora in the saliva and on the oral mucosal surfaces of patients with OPMD.

Lichen planus (LP) is a chronic, idiopathic, inflammatory disease of the oral mucosa or the skin, presenting as a white lesion when it affects the oral cavity (oral lichen planus, OLP). A crucial aspect of the pathomechanism of OLP is the accumulation of CD8+ T lymphocytes under the basal cell layer of the oral mucosa, which causes DNA damage and keratinocyte apoptosis by antigen-specific cell-mediated immune response. CD8+ T cells degrade the basement membrane as well. According to the most accepted hypothesis, chronic stimulation from inflammatory and stromal cells provides the initial signal which leads to the uncontrolled growth of epithelial cells. Additionally, oxidative stress induced DNA damage could also lead to neoplastic changes, but the initial event leading to this signal cascade activation has not been characterized yet. Based on several lines of evidence, viral, fungal, and bacterial antigens have all been suggested as a potential initiating factor in LP. If there is a relationship between the bacterial flora and OLP, the question is whether the trigger area is in the oral cavity, or at another area of the body, such as the skin and the genitals, the gastrointestinal tract, the larynx or the eyes. If oral bacterial infection could initiate OLP development, it is not clear whether a single bacterial species could initiate the OLP transformations, or the interaction of several species elicits this process. Additionally, the disturbed balance of the normal bacterial flora could also be involved in the initial steps of OLP activation. Based of the above, we have investigated the oral mucosal microbial colonisation of our patients. We have found that the OPMD showed altered bacterial composition compared to the healthy mucosa.

2 Objectives

The general objective was to investigate the microbiome and microbiota of the OPMD with unknown etiology.

Our specific objectives were:

1. To analyse and compare the microbiome of the clinically healthy and OPMD-affected oral mucosal tissues by metagenome sequencing technique.

   We hypothesized, that the microbiome of clinically healthy and OPMD-affected tissues are different, i.e. pathogenic bacteria are overrepresented in the mucosal samples of OPMD affected tissues.

2. To analyse and compare the microbiota of the healthy and OPMD oral mucosal tissues by MALDI-TOF MS analysis of cultured bacteria.

   We hypothesized, that the data obtained by MALDI-TOF MS will be comparable to the results of metagenome sequencing.

3. To verify inflammatory histopathological changes after the mucosal infiltration by *H. pylori*, a bacterium of known tumorigenic potential, in an *in vivo* murine experimental model.

   We hypothesized, that if OPMDs are caused or triggered by bacteria, the histopathological changes in OPMD lesions may be similar to the changes in *H.pylori* induced gastric mucosal inflammation and cancer.

4. To compare our results with existing research.

   We hypothesized, that the bacterial alterations in OPMD lesions will be comparable those suggested by the current literature for OSCC.

3 Materials and Methods

In our experimental setup, we examined the oral microbiome and microbiota of patients diagnosed with OPMD. To make the pathological diagnosis, hematoxylin and eosin stained sections were made from punch biopsy specimens of affected mucosal area. We compared the microbiome and microbiota of healthy and non-healthy mucosal surfaces. To gain comparable data, we applied two different methods: the metagenome sequencing of total DNA purified from tissue specimens and MALDI-TOF MS analysis from cultured bacteria. We found more differences besides the overlapping results of two methods. Although *Fusobacterium nucleatum* was detected by metagenomic sequencing in OPMD biopsies, it could not be detected by MALDI-TOF in OPMD swab samples cultivated *in vitro*. To resolve this discrepancy, we made qPCR identification of *Fusobacterium nucleatum* - a bacterium implicated in carcinogenesis. Colony forming units (CFUs) were calculated by comparing the means of thresold cycles to those of serial dilution samples of control *F. nucleatum* cultures. Finally, we used an experimental murine model to study mucosal lesions elicited by *Helicobacter pylori* and compared the histopathological alterations observed to the OPMD affected mucosa. We performed light-microscopic evaluation of hematoxylin-eosin and Giemsa stained sections.

4 Results

4.1 Metagenome sequencing

There were no significant differences in the distributions of taxonomic domains between the healthy tissue and the OPMD lesion. 6.78% of bacterial domains were detected in healthy area compared 6.87% of bacterial domains in white lesions of oral mucosa. Metagenome sequencing revealed that the bacterial diversity in the OPMD biopsies was higher compared to the healthy oral mucosa. Within the Bacteria domain *Firmicutes, Fusobacteria, Proteobacteria, Actinobacteria, Bacteroidetes* phyla were present in the healthy oral mucosa. In the OPMD biopsies the same phyla were identified in descending order of relative abundance; however, two additional phyla, *Fibrobacteres* and *Spirochaetes* were observed, too. Metagenome sequencing detected 18 different bacterial species in healthy tissue and 43 species in the OPMD lesion, whereas 8 bacterial species were detected in both samples. The ratio of Streptococci to all oral bacteria was not significantly different in the healthy tissue

compared to the OPMD lesion. The relative abundance of *Streptococcus mitis* dramatically decreased in OPMD, as compared to the healthy tissue. The ratio of *Fusobacterium nucleatum* was markedly increased in the OPMD lesions, as compared to the healthy tissue detected by metagenome sequencing.

4.2 MALDI-TOF MS

MALDI-TOF MS analysis detected 41 different bacterial species in healthy tissue and 36 species in the lesion. 25 bacterial species were detected in both samples. In metagenome sequencing detected *Fusobacterium nucleatum* was not presented in OPMD samples.

4.3 *Fusobacterium nucleatum* qPCR of healthy oral mucosa and the OPMD samples.

In vitro bacterial cultures of oral swab samples from healthy mucosa and OPMD lesions (used for MALDI-TOF MS analysis) were assayed using *Fusobacterium nucleatum*-specific qPCR. OPMD showed a significantly higher colonization by *F. nucleatum* compared to healthy mucosa.

4.4 Murine model of mucosal lesion caused by bacterial infection

Long-term *H. pylori* infection of positive control (infected, untreated) mice show *H. pylori* foveolar colonization in gastric fundus sample, and the intra-epithelial mononuclear lymphoid elements of the mucosa are noted. We did not detect lichenoid infiltration of lymphoid cells, in contrast to the histological findings described in the case of lichen, or in several cases of verrucous leukoplakias in humans.

5 Discussion

Actinomyces spp., Veilonella spp., Fusobacterium spp., Prevotella spp., Streptococcus spp., and *Corynebacterium spp*. were represented in our OPMD samples, similarly to OSCC with mentioned putative role in carcinogenesis. *Gemella spp*. as a suggested spp with putative role in OSCC carcinogenesis was found also in our OPMD samples, but was represented in higher

rate in healthy mucosa. We did not find in our OPMD samples bacteria characteristic for OSCC, such as *Serratia liquefaciens, Klebsiella pneumoniae, Citrobacter freundii, Enterococcus faecalis, Propionibacterium spp., Clostridium spp., Porphyromonas spp., Bacteroides ureolyticus/gracilis, Clavibacter michigenensis, Ralstonia insidosa, Peptostreptococcus stomatis, Johnsonella ignova, Enterobacteriaceae (family), Tenericutes (phylum), Stomatoccus spp.*

Regarding the putative role of distinct oral bacteria in tumorigenesis, the association of *Fusobacterium nucleatum* with colorectal carcinoma and oral carcinoma was documented. Moreover, it was suggested that a distinct subspecies of *F. nucleatum*, such as *F. nucleatum subsp. polymorphum* may play an etiopathogenetic role in oral carcinogenesis. We observed that the relative abundance of *Streptococcus mitis* decreased dramatically in the pathological niche of OPMD. Since *Streptococcus species* are characteristic components of the oral flora, the quantitative analysis of these bacteria is indispensable for the understanding of pathological processes. Streptococci comprise of a wide variety of bacterial species that interact with other members of the oral microbiome. It was suggested that *S. mitis* is involved in the maintenance of a healthy oral flora by affecting adhesion and biofilm formation by periodontal pathogens to. Decreased relative abundance of *S. mitis* and an increased relative abundance of *F. nucleatum* may play a role in the transition of OPMDs to oral cancer.

In contrast to the sequencing data, overrepresentation of *Fusobacterium nucleatum* was not detected in the OPMD samples with MALDI-TOF MS. Detection of *Fusobacterium nucleatum* by qPCR in the very same samples that were used for MALDI-TOF analysis revealed that the sensitivity and specificity of culturing followed by MALDI-TOF MS examination for *F. nucleatum* was in our case low. The results obtained from the different methods used indicated that metagenome sequencing yielded a more diverse bacterial community in OPMD than MALDI-TOF MS.

The effect of **mucosal inflammation** caused by immunresponse of immune cells against bacteria may also contribute to tumorigenesis. Histopathologically, we can examine the presence of mucosal histologycal and cellular alterations, accumulation of inflammatory cells and bacterial invasion.

To compare the histopathological inflammational signs of our OPMD tissue samples, we checked a known mucosal infection wich increase the risk of malignant transformation - inflamed gastric mucosa of *Helicobacter pylori* infected mice. In the murine model, there

were numerous of mononuclear lymphoid elements in the inflamed mucosa. That chronic inflammation may lead to formation of **tumorigenic microenvironment**. In OPMD there are both supporting as well as negative evidences for a connection with *H. pylori* infection. **In our animal model we detected the signs of inflammation in *H. pylori* infected gastric mucosa**.

6 Conclusion

Although our work is not suitable to answer the „Chicken or the Egg" problem – that the suggested microbial changes are cause or effect of OPMD - in all aspects, but we have found that the bacterial colonization of mucosa has already altered in OPMD tissues. Some alterations correlate with results of the studies about the putative role of bacteria in OSCC development. Our most conspicouos finding was that *Fusobacterium nucleatum* is overrepresented in OPMD tissue samples. *Fusobacterium nucleatum* was implicated in malignant transformation of mucosal cells of gastrointestinal tract, especially in the colon, and several studies suggest a similar role for *F. nucleatum* and other pathogenic bacteria in oral mucosal cells.

1. We found that the microbiome of clinically healthy and **OPMD** affected oral tissues are **different**: pathogenic bacteria were overrepresented in the mucosal samples of OPMD affected tissues. Our results showed that ***Fusobacterium nucleatum***, a bacterium implicated in tumorigenesis, is overrepresented in the samples from OPMD affected regions. In addition, we observed an increased bacteral diversity in OPMD, compared to the microbiota of healthy oral mucosa. These observations may form the basis of novel therapeutic approaches preventing oral carcinogenesis in a subset of patients with OPMD.

2. The comparison of MALDI-TOF MS and metagenome sequencing provided **partially overlapping results.**

3. We found inflammatory signs both in human OPMD samples and in an *in vivo* murine model of *H. pylory*-infected gastric mucosa. **This may indicate that one can't exclude a potential role for *H. pylori* infection or inflammation elicited by other oral bacteria could play a role in the transition of OPMD to carcinoma in humans.**

4. Comparison of the microbiome of OPMD samples analysed in our study and the microbiome/microbiota of OSCC lesions reviewed in the literature revealed that a wide variety of non-overlapping sets of bacterial taxa were found to be associated with OSCC lesions. ***Fusobacterium nucleatum*** **emerged from the data as the best potential candidate contributing to the transition of OPMD to OSCC.**

6.1 Summary of accomplished objectives

1. Pathogenic bacteria are overrepresented in the mucosal samples of OPMD affected tissues.

2. Specificity and sensitivity of metagenome sequencing and cultivation based MALDI-TOF MS is markedly different to oral bacteria.

3. *Helicobacter pylori* caused gastric inflammation in mice could be an experimental model of bacterial inflammation triggered carcinogenesis of oral mucosal surfaces. (Keeping the limitations caused by differences between subjects and modellorganisms in mind.)

4. *Fusobacterium nucleatum* emerged from the data as the best potential candidate contributing to te transition of OPMD to OSCC.

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