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ORIGINAL ARTICLE

Chicken or the Egg: Microbial Alterations in Biopsy Samples of Patients with Oral Potentially Malignant Disorders

Gabor Decsi[1] · Jozsef Soki[2] · Bernadett Pap[3] · Gabriella Dobra[3] · Maria Harmati[3] · Sandor Kormondi[4] · Tibor Pankotai[5] · Gabor Braunitzer[6] · Janos Minarovits[7] · Istvan Sonkodi[1] · Edit Urban[2] · Istvan Balazs Nemeth[8] · Katalin Nagy[1] · Krisztina Buzas[3,7]

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Abstract

Oral carcinogenesis often leads to the alteration of the microbiota at the site of the tumor, but data are scarce regarding the microbial communities of oral potentially malignant disorders (OPMDs). Punch biopsies were taken from healthy and non-healthy mucosa of OPMD patients to analyze the microbiome using metagenome sequencing. In healthy oral mucosa biopsies the bacterial phyla *Firmicutes, Fusobacteria, Proteobacteria, Actinobacteria* and *Bacteroidetes* were detected by Ion Torrent sequencing. The same phyla as well as the phyla *Fibrobacteres* and *Spirochaetes* were present in the OPMD biopsies. On the species level, there were 10 bacterial species unique to the healthy tissue and 35 species unique to the OPMD lesions whereas eight species were detected in both samples. We observed that the relative abundance of *Streptococcus mitis* decreased in the OPMD lesions compared to the uninvolved tissue. In contrast, the relative abundance of *Fusobacterium nucleatum*, implicated in carcinogenesis, was elevated in OPMD. We detected markedly increased bacterial diversity in the OPMD lesions compared to the healthy oral mucosa. The ratio of *S. mitis* and *F. nucleatum* are characteristically altered in the OPMD lesions compared to the healthy mucosa.

Keywords OPMD · Oral microbiome · Metagenome sequencing · Lichen · Leukoplakia

✉ Krisztina Buzas
kr.buzas@gmail.com; buzas.krisztina@brc.mta.hu

[1] Faculty of Dentistry, Department of Oral Surgery, University of Szeged, Tisza Lajos krt. 64, Szeged H-6720, Hungary

[2] Albert Szent-Gyorgyi Clinical Centre, Institute of Clinical Microbiology, University of Szeged, Semmelweis u. 6, Szeged H-6725, Hungary

[3] Biological Research Centre, Hungarian Academy of Sciences, Temesvari krt. 62, Szeged H-6726, Hungary

[4] Albert Szent-Gyorgyi Clinical Centre, Department of Traumatology, University of Szeged, Semmelweis u. 6, Szeged H-6725, Hungary

[5] Department of Biochemistry and Molecular Biology, University of Szeged, Kozep fasor 52, Szeged H-6726, Hungary

[6] dicomLAB Ltd., Pulz u. 46/b, Szeged H-6724, Hungary

[7] Faculty of Dentistry, Department of Oral Biology and Experimental Dental Research, University of Szeged, Tisza Lajos krt. 64, Szeged H-6720, Hungary

[8] Department of Dermatology and Allergology, University of Szeged, Szeged H-6720, Hungary

Introduction

Numerous scientific data demonstrate the altered bacterial colonization of cancerous tissue, but the causality of microbial alteration has not clarified yet.

The role of oral microbes in the development of oral potentially malignant disorder (OPMD) and oral squamous cell carcinoma (OSCC) is periodically reevaluated, since OPMD or OSCC may develop in the absence of the traditional risk factors like smoking, alcohol consumption and betel nut use [1–3]. While the microbiological background of oral squamous cell carcinoma was intensively studied in the last two decades, less attention has been paid to OPMD in this respect [4].

OPMD is a group of disorders of diverse etiologies, frequently associated with tobacco consumption and mutations in the DNA of oral epithelial cells. A fraction of OPMD undergoes clinical and histomorphological alterations that lead to the development of OSCC. These disorders include leukoplakia, erythroplakia, oral lichen planus, submucous fibrosis, and actinic cheilitis [5, 6]. In addition, inherited cancer syndromes such as xeroderma pigmentosum and Fanconi's

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anemia are also associated with an increased incidence of malignant tumors, among them oral carcinoma [6].

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 [6]. 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 [7]. 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 in the anamnesis. Distinct histopathological changes, like hyperkeratosis with or without dysplasia, may accompany the transition of PVL to verrucous hyperplasia and verrucous carcinoma [6].

There are conflicting results regarding the association of leukoplakia and human papillomavirus (HPV) infection [6, 8]. The role of torque teno virus (TTV) [9, 10], Epstein-Barr virus (EBV) [11] and *Candida albicans* [8, 12–16] in leukoplakia development and carcinogenesis remains to be clarified, too. Additionally, it has been also demonstrated that specific bacterial infections like *Helicobacter pylori* [17] or the intracellular *Mycoplasma salivarium* [18] could also be involved in this process.

A disbalance in the oral microbial flora can be also accompanied with leukoplakia and carcinogenesis, as suggested by the association of periodontal inflammation with leukoplakia [19] and a changing bacterial flora in the saliva and on the oral mucosal surfaces of patients with OPMD [20, 21].

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 the apoptosis of the keratinocytes by antigen-specific cell-mediated immune response, and also basement membrane degradation [22–25]. According to the most accepted hypothesis, chronic stimulation from the inflammatory and stromal cells provides the initial signal which lead to the abolished growth control 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 the increasing evidence viral, fungal, and bacterial antigens have all been suggested [26–34] 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, 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 is it the interaction of several species during the process? Additionally, the disturbed balance of the normal bacterial flora could also be involved in the initial steps of OLP activation.

In our experimental setup, we examined the oral microbiome of patients diagnosed with OPMD. We compared the microbiome of healthy and non-healthy mucosal surfaces. Using metagenome sequencing, we detected markedly increased bacterial diversity in the OPMD lesions compared to the healthy oral mucosa. In parallel, in the OPMD lesions there was a reduced representation of distinct *Streptococcus* species that dominate the bacterial community of healthy oral mucosa.

Materials and Methods

Patient Selection

Potential participants were interviewed by the clinical coordinators at the University of Szeged, Faculty of Dentistry. Every potential participant was informed about the ethical permission of the study and received both written and oral information on the goals and procedures of this study. The initial participants were selected by volunteering activity and the detailed questionnaire on family anamnesis, chronic diseases, medication-, alcohol-, tobacco- and drug consumption, oral hygiene, and sexual habits was filled out by the volunteers. Eligibility was determined based on the results of this questionnaire. Female and male subjects over 18 years of age were eligible for the study, provided that they were able to provide signed and dated informed consent and if they did not meet any of the exclusion criteria. As for the patient group, an established diagnosis of OPMD was also a requirement. The exclusion criteria included vital signs outside the acceptable range at the screening visit (i.e., blood pressure > 140/90 mmHg, oral temperature > 37 °C, heart rate > 100/min), pregnancy, the potential subject being a sex worker, topical antibiotic treatment up to 7 days before the screening visit, and the use of the following drugs within 2 months before the screening visit: systemic antibiotics, corticosteroids, cytokines, methotrexate or immunosuppressive cytotoxic agents, or large doses of commercial probiotics ($\geq 10^8$ CFU mL^{-1} organisms per day). No patients were on specific diet, nor on antibiotic therapy in the previous 6 months. The clinical characteristics and brief medical history of the patient group is given in Table 1.

The study protocol conformed to the Declaration of Helsinki in all respects and was approved by the Institutional Ethics Committee of the University of Szeged (No. 3161).

Table 1 The brief medical history of patients

patient #	1	2	3	4	5	6	7
sex	♂	♂	♀/2 PM	♀/2 PM	♂	♂	♀/1 PM
age	60	73	62	57	85	54	77
clinical diagnosis	leukoplakia verrucosa labii	leukoplakia proliferativa verrucosa gingivae	lichen reticularis et atrophicans buccae et linguae	lichen reticularis et hypertrophicus buccae	lichen reticularis buccae	lichen atrophicans buccae	lichen reticularis et atrophicans buccae et gingivae
pathological diagnosis	OPMD - verrucous hyperplasia	OPMD – lichenoid gingivitis with hyperplasia, reactive changes, together with basal cell dysplasia	OPMD – lichenoid buccitis with hyperplasia and reactive changes	OPMD – lichenoid buccitis with hyperplasia and reactive changes	OPMD – lichenoid buccitis with reactive changes	OPMD – lichenoid buccitis with massive hyperplasia and reactive changes	OPMD – lichenoid buccitis with hyperplasia and reactive changes
systemic diseases	HT, onychomycosis, MI, cGa, cDu, IHD	HT, hChol, hTG	no	HT	HT, hypothyr., h.uricemia	no	HT, DM, RA, h.uricemia, hyperthyr., hChol
allergy	no	no	no	no	medicine allergy	no	no
pack years of smoking	10	0	0	0	20	20	2.5
alcohol consumption/day	no	2–3 unit (wine)	no	no	2 unit (wine)	no	no
frequency of tooth- brushing	every week	many times a day	many times a day	many times a day	once a day	many times a day	many times a day
use of oral rinse	no	many times a day, changed	no	Listerine/ Corsodyl	no	Listerine/ Meridol	Listerine
sexual activity	none in the past 4 yrs	active	active	active	active	active	active
history of oral sexual activity	no	no	no	yes	no	yes	no
medications at the time of the study:	Asactal, Concor, Pantoprazol, Roxera, Apranax, Tritace	Lipidil, Xeter, Chinotal, Nortivan, Agen, Aspirin Protect	Ø	Betaserc, Nootropil, Prenessa, Frontin	Tiroxin, Milurit, Warfarin	Ø	1

Abbreviations: OPMD: oral potentially malignant disorder; PM: postmenopausal; /1/2: parity; HT: hypertension; MI: Myocardial infarct; cGa: chronic gastritis; cDu: chronic duodenitis; IHD: ischemic heart disease; hChol: hypercholesterinaemia; hTG: Hypertrigliceridaemia, DM: diabetes mellitus; RA: rheumatoid arthritis; hypothr: hypothryreosis, hyperthr:hyperthyreosis, h.uricemia: hyperuricemia

[1] L-Thyroxin, Meloxep, Rosuvastatin, Glucobay, Milurit, Talliton, Frontin, Tramadol, Moxogamma, Metoprolol, Enalapril, Meloxep, Apo-Famotidin, chrom, vitamin-C, vitamin-D, Mg-B6, kurkuma

Table 2 The histopathological characteristics of the biopsies

patient #	1	2	3	4	5	6	7
OPMD	+	+	+	+	+	+	+
hyperkeratosis	+	++	+++	+++	+	+++	+++
hyperplasia	+++	++	+	+++	–	+++	+++
reactive atypia	–	+	–	+	–	+	+
dysplasia	–	+	–	–	–	–	–
lichenoid infiltrate	+	+++	++	+++	+++	+++	++
MT	–	–	–	–	–	–	–

Abbreviations: (− no, + mild, ++ moderate, +++ severe; OPMD: oral potentially malignant disorder; MT: malignant transformation)

Tissue Sample Collection for Metagenome Sequencing, Histopathological Assessment

As a classical method for tissue sample collection from mucosal lesions in the oral cavity, we used punch biopsy. The biopsy was made with punches 4 mm in diameter. We collected 2 identical samples from the intralesional area: one sample was taken for hematoxylin-eosin (HE) staining, and an additional sample for metagenome sequencing. Additionally, we also collected a third, control specimen from the ipsilateral healthy mucosa for metagenome sequencing. The specimens contained the mucosa, and submucosal connective tissue. Samples for metagenome sequencing were collected in Eppendorf tubes filled with 20 mM potassium phosphate buffer (pH 7.0), and stored at −20 °C. Tissue samples for HE staining were fixed in formaldehyde solution, and sent for histopathological assessment. Pathological dataset (Table 2) included the absence or presence, together with severity of hyperkeratosis, hyperplasia, reactive atypia, dysplasia, and lichenoid infiltrate [35].

Metagenomic DNA Isolation

Total DNA was isolated from patient samples as described previously, with minor modifications [36]. Briefly, samples (600 μL) were suspended in 650 μL of extraction buffer (100 mM TrisCl, pH 8.0, 100 mM EDTA, pH 8.0, 1.5 M NaCl, 100 mM sodium phosphate, pH 8.0, 1% CTAB) 3.5 μL proteinase K (20.2 mg mL^{-1}) and incubated horizontally at 37 °C for 45 min, next 80 μL of 20% SDS was added and mixed by inversion for several times with further incubation at 60 °C for 1 h. The sample in each tube was mixed thoroughly every 15 min. The particles were collected by centrifugation (17,000 g) for 5 min. The supernatant was transferred into clean tubes and was mixed with equal quantity of phenol chloroform and isoamyl alcohol (25:24:1) and extracted three times. DNA was precipitated with 0.7 volume isopropanol, the pellet was washed with 70% ethanol. Crude DNA pellets were dried and dissolved in 50 μL of TE buffer (10 mM Tris-HCl, 1 mM sodium EDTA, pH 8.0). Metagenomic DNA was quantified using Qubit® 2.0 Fluorometer. Half of total metagenomic DNA from the

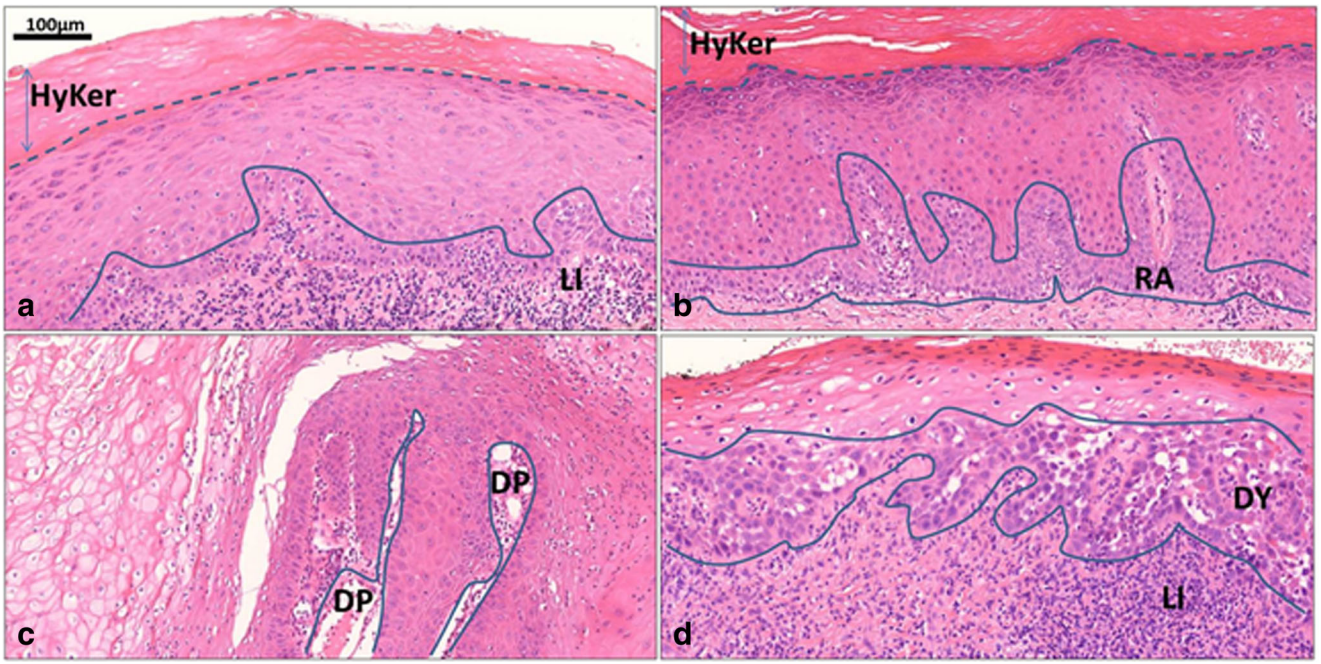

Fig. 1 Representative pictures from OPMD tissue samples OPMD tissue samples with marked hyperkeratosis (**a, b** – HyKer), acanthosis and lichenoid infiltrate (**a, d** – LI). Occasionally there was a marked verrucous hyperplasia with elongated dermal papillae (**c** – DP). Note the regenerative basal cell changes (**b** – RA/regenerative atypia/), in contrast to mild dysplasia (**d** – DY) with focal acantholysis /HE; OM 112×/

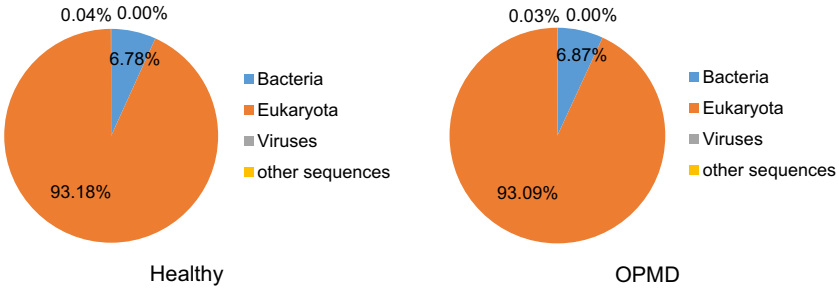

Fig. 2 Distribution of taxonomic domains Significant differences were not found in the distributions of taxonomic domains between the healthy tissue and the OPMD lesion. 6.78% of bacterial domains were detected in healthy area compared 6.87% of bacterial domains in white lesions of oral mucosa

healthy and lesion samples were pooled and stored at −20 °C for sequencing.

Library Preparation and Sequencing

Ion Torrent PGM Fragment libraries of 200 nt were generated according to the appropriate protocols (Ion Torrent PGM, Life Tech, USA). 1 μg pooled metagenomic DNA from each sample was used for library preparation for each sample. DNA shearing and end-repair was achieved by Ion Xpress™ Plus Fragment Library Kit, and DNA Purification was carried out by PureLink PCR Purification Kit (Thermo Fisher Scientific, MA). Adapter ligation and nick translation were performed by Ion Shear Plus Reagents Kit (Thermo Fisher Scientific, MA). Size selection was performed in 2% agarose gel to enrich the 300–350 nt fragments then library amplification was achieved by using Platinum® PCR SuperMix (Thermo Fisher Scientific, MA). ION Library TaqMan qPCR was used for quantitation and Ion Xpress Template 200 ePCR kit was used for the emulsion PCR. Sequencing was performed on Ion Torrent Personal Genome Machine™ using Ion 318 chip. Ion Torrent Personal Genome Machine™ sequencing resulted 872,798 sequence reads for sample 1 (control) with an average read length of 219 ± 71 bases and 644,200 sequence reads for sample 2 (diseased) with an average read length of 220 ± 72 bases.

Quality Assurance

The MG-RAST software performs a QC (quality control) and an automatic normalization of the FASTQ sequence. For the taxonomical analyses, maximum e-value cut-off of 10^{-15}, minimum percent identity cut-off 90% and minimum alignment length cut-off 40 nt settings were applied. The overall community composition was determined using the M5nr database. Hits for the eukaryotic data were removed and relative abundance of the bacterial data was calculated.

Swab Sample Collection

We used cotton swabs for collecting bacterial samples. Two individual samples were taken from each patient, one from the surface of the lesion, and another one from the ipsilateral healthy (non-involved) mucosa. The swabs were rolled 4 times over the chosen areas. Then the swabs were placed in anaerobic

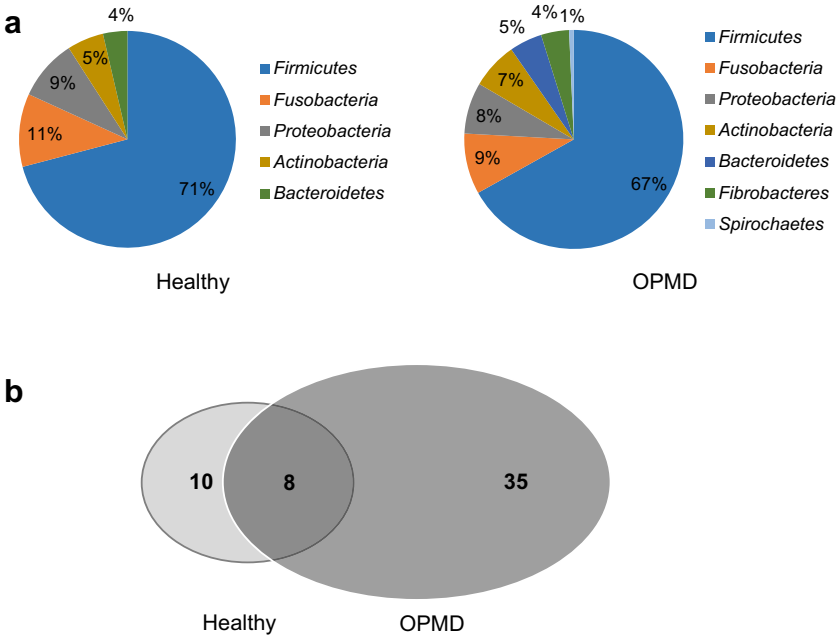

Fig. 3 Bacterial diversity a Metagenome sequencing revealed that the bacterial diversity in the OPMD biopsies was higher compared to the healthy oral mucosa. Within the Bacteria domain *Firmicutes, Fusobacteria, Proteobacteria, Actinobacteria, Bacteroidetes* phyla were present in the healthy oral mucosa. 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. **b** Metagenome sequencing detected 18 different bacterial species in healthy tissue and 43 species in the OPMD lesion. Eight bacterial species were detected in both samples

transport medium (AnaerobeSystems, CA) and sent immediately to the Institute of Clinical Microbiology, University of Szeged, for *Fusobacterium nucleatum* cultivation.

Fusobacterium nucleatum qPCR

DNA templates for qPCR were prepared from the undiluted BHI suspensions of the oral swabs by the QiAmp Stool Mini DNA Kit (Qiagen, Germany) as recommended by the supplier. Subsequent quantitative RT-PCRs for *Fusobacterium nucleatum* were done in StepOne RT-PCR instrument (Invitrogen, CA) using 5 μL Brilliant II master mix (Agilent), primers FnucF CTTAGGAATGAGACAGAGATG and FnucR TGATGGTAACATACGAAAGG 0.2 μL (35 pmole/μL) each, 1 μL of template sample and the following cycling conditions: starting denaturation and hot start activation 95 °C 10 min, 95 °C 15 s, 56 °C 15 s and 72 °C 30 s, 40×; and a melting curve from 72 °C to 95 °C [37] CFUs were calculated comparing the means of threshold cycles to ones of a *F. nucleatum* 10-fold serial dilution samples prepared with the same kit.

Data Analysis

For the comparison of the numerical data, the Mann-Whitney U test was used in SPSS 21.0 (IBM, NY).

Results

Sample selection was based on the histological analysis of oral potentially malignant disorder. Figure 1 shows representative pictures of the OPMD tissue samples with marked hyperkeratosis, acanthosis and lichenoid infiltration. Table 1. describes the brief medical history of selected patients (Selection criteria has been found in Materials and methods). Detailed histological classification summarized in Table 2.

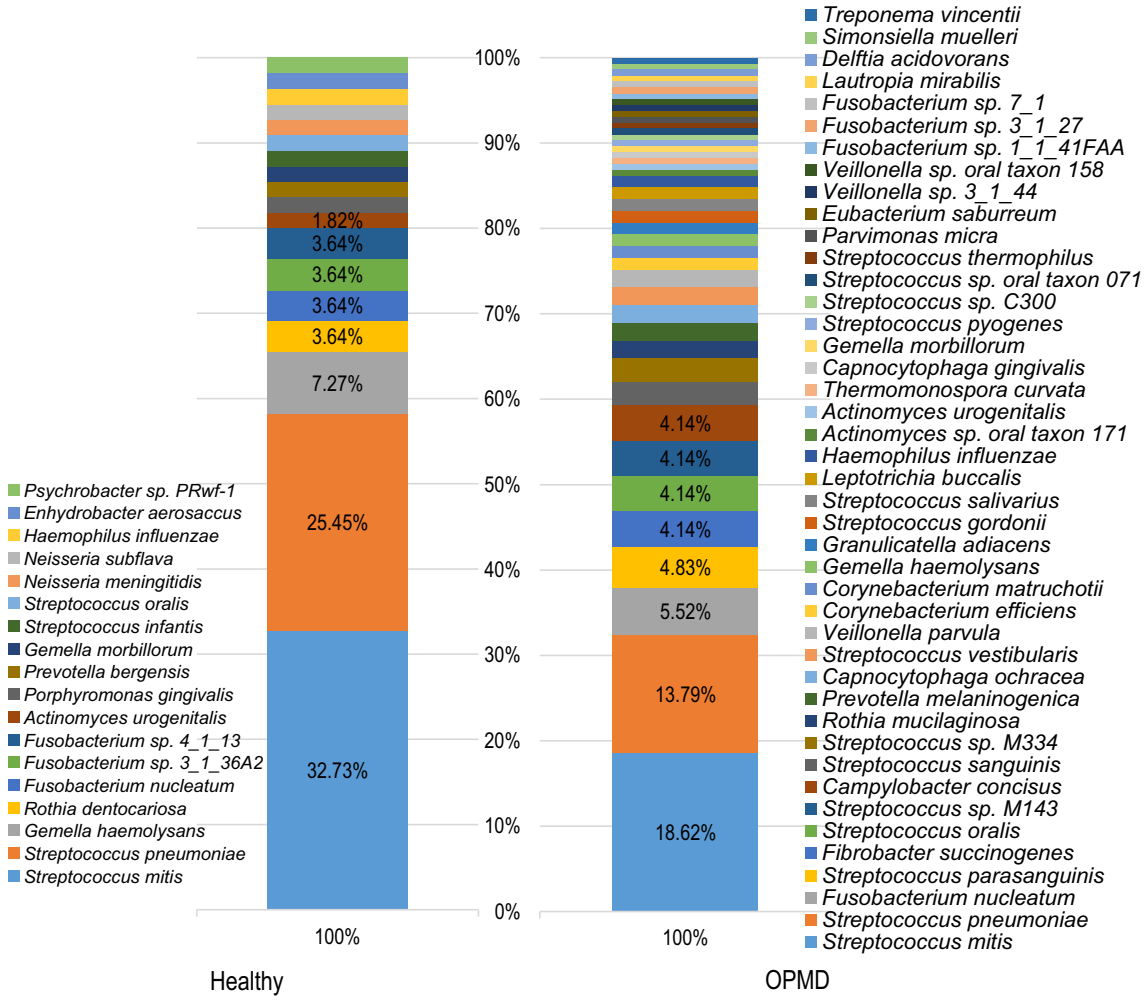

Fig. 4 Comparison of the detected bacterial species Metagenome sequencing detected 18 different bacterial species in healthy tissue and 43 species in the OPMD lesion

First, we determined the ratio of bacterial DNA in our sample. As punch biopsy samples contain a high amount of human tissue, this step helped us to define the limitation of the study. In healthy oral mucosa samples, 6.78% of the sequence reads were annotated to the domain *Bacteria*. The ratio of bacterial sequences was similar (6.87%) in the pooled OPMD DNA samples (Fig. 2).

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, i.e. *Firmicutes, Fusobacteria, Proteobacteria, Actinobacteria and Bacteroidetes* were identified in descending order of relative abundance; however, two additional phyla, *Fibrobacteres* and *Spirochaetes* were observed, too (Fig. 3a).

Table 3 Comparison of bacterial diversity in the healthy mucosa and the OPMD lesion

Species	Phylum	Healthy	OPMD
Streptococcus mitis	Firmicutes	32,73%	18,62%
Streptococcus pneumoniae	Firmicutes	25,45%	13,79%
Gemella haemolysans	Firmicutes	7,27%	1,38%
Rothia dentocariosa	Actinobacteria	3,64%	0,021
Fusobacterium nucleatum	Fusobacteria	3,64%	5,52%
Actinomyces urogenitalis	Actinobacteria	1,82%	0,69%
Streptococcus oralis	Firmicutes	1,82%	4,14%
Haemophilus influenzae	Proteobacteria	1,82%	1,38%
Fusobacterium sp. 3_1_36A2	Fusobacteria	3,64%	0
Fusobacterium sp. 4_1_13	Fusobacteria	3,64%	0
Porphyromonas gingivalis	Bacteroidetes	1,82%	0
Prevotella bergensis	Bacteroidetes	1,82%	0
Gemella morbillorum	Firmicutes	1,82%	0
Streptococcus infantis	Firmicutes	1,82%	0
Neisseria meningitidis	Proteobacteria	1,82%	0
Neisseria subflava	Proteobacteria	1,82%	0
Enhydrobacter aerosaccus	Proteobacteria	1,82%	0
Psychrobacter sp. PRwf-1	Proteobacteria	1,82%	0
Streptococcus parasanguinis	Firmicutes	0	4,83%
Fibrobacter succinogenes	Fibrobacteres	0	4,14%
Streptococcus sp. M143	Firmicutes	0	4,14%
Campylobacter concisus	Proteobacteria	0	4,14%
Streptococcus sanguinis	Firmicutes	0	2,76%
Streptococcus sp. M334	Firmicutes	0	2,76%
Prevotella melaninogenica	Bacteroidetes	0	2,07%
Capnocytophaga ochracea	Bacteroidetes	0	2,07%
Streptococcus vestibularis	Firmicutes	0	2,07%
Veillonella parvula	Firmicutes	0	2,07%
Rothia mucilaginosa	Actinobacteria	0	2,07%
Corynebacterium efficiens	Actinobacteria	0	1,38%
Corynebacterium matruchotii	Actinobacteria	0	1,38%
Granulicatella adiacens	Firmicutes	0	1,38%
Streptococcus gordonii	Firmicutes	0	1,38%
Streptococcus salivarius	Firmicutes	0	1,38%
Leptotrichia buccalis	Fusobacteria	0	1,38%
Actinomyces sp. oral taxon 171	Actinobacteria	0	0,69%
Thermomonospora curvata	Actinobacteria	0	0,69%
Capnocytophaga gingivalis	Bacteroidetes	0	0,69%
Gemella morbillorum	Firmicutes	0	0,69%
Streptococcus pyogenes	Firmicutes	0	0,69%
Streptococcus sp. C300	Firmicutes	0	0,69%
Streptococcus sp. oral taxon 071	Firmicutes	0	0,69%
Streptococcus thermophilus	Firmicutes	0	0,69%
Parvimonas micra	Firmicutes	0	0,69%
Eubacterium saburreum	Firmicutes	0	0,69%
Veillonella sp. 3_1_44	Firmicutes	0	0,69%
Veillonella sp. oral taxon 158	Firmicutes	0	0,69%
Fusobacterium sp. 1_1_41FAA	Fusobacteria	0	0,69%
Fusobacterium sp. 3_1_27	Fusobacteria	0	0,69%
Fusobacterium sp. 7_1	Fusobacteria	0	0,69%
Lautropia mirabilis	Proteobacteria	0	0,69%
Delftia acidovorans	Proteobacteria	0	0,69%
Simonsiella muelleri	Bacteroidetes	0	0,69%
Treponema vincentii	Spirochaetes	0	0,69%

On the species level, 18 bacterial species were detected in the healthy tissue, 35 species were unique for the OPMD lesions and eight bacterial species were detected both in healthy oral mucosa and the OPMD biopsy samples (Fig. 3b). Metagenome sequencing showed markedly increased bacterial diversity in the OPMD tissue samples. Analysis of bacterial species detected 2.38 fold increasing in diversity of the OPMD lesions (Fig. 4, Table 3).

Based on the data of metagenome sequencing, the relative abundance of *Streptococcus* sp. did not show significant difference between the healthy tissue and the OPMD lesions: it was 61.82% in healthy tissue and 58.62% in OPMD, respectively (Fig. 5a) However, the relative abundance of the *Streptococcus mitis* was found to be dramatically decreased in OPMD: it was only 18.62%, compared to 32.73% in the healthy tissue (Fig. 5b). Moreover, examining all *Streptococcus* species their prevalence in the OPMD lesions was determined to be more diverse compared to the healthy tissue (Table 3).

According to the results of metagenome sequencing, the ratio of *Fusobacterium nucleatum* was higher in the OPMD lesions (5.52%), compared to the healthy tissue (3.64%) (Fig. 6). Relative abundance of *Fusobacterium nucleatum* (5.52%) and *Streptococcus oralis* (4.14%) bacteria were identified in a larger portion among the overlapping bacteria in OPMD while 3.64 and 1.82% were observed in healthy tissue, respectively). The abundances of the species *Gemella haemolysans* (1.38%), and the above mentioned *S. mitis* (18.62%) decreased markedly in OPMD as compared to the healthy tissue (7.27% and 32.73%) (Table 3).

35 different bacterial species has been found in the OPMD lesions which has not been presented in the healthy tissue, e.g., *Streptococcus parasanguinis, Fibrobacter succinogenes, Campylobacter concisus, Streptococcus sanguinis, Rothia mucilaginosa, Prevotella melaninogenica, Capnocytophaga ochracea, Streptococcus vestibularis, Veillonella parvula, Corynebacterium efficiens, Corynebacterium matruchotii, Granulicatella adiacens, Streptococcus gordonii, Streptococcus salivarius, Leptotrichia buccalis, Thermomonospora curvata, Capnocytophaga gingivalis, Streptococcus pyogenes Streptococcus thermophiles, Parvimonas micra, Eubacterium saburreum, Lautropia mirabilis, Delftia acidovorans, Simonsiella muelleri, Treponema vincentii,* etc., in descending order of the abundances.

As *F. nucleatum* was implicated in carcinogenesis in several cancers [38], we verified the results of metagenome sequencing by *F. nucleatum*-specific qPCR. As Fig. 7 shows, the number of bacteria (CFUs) is significantly higher ($P < 0.0001$) in samples taken from OPMD lesions than in those from the healthy mucosa. This difference is remarkable, even when one considers the high inter-individual variability of the samples.

Discussion

It was suggested that OPMD was frequently associated with alcohol and tobacco consumption, and affected mainly males in their fifties, but the practice shows that

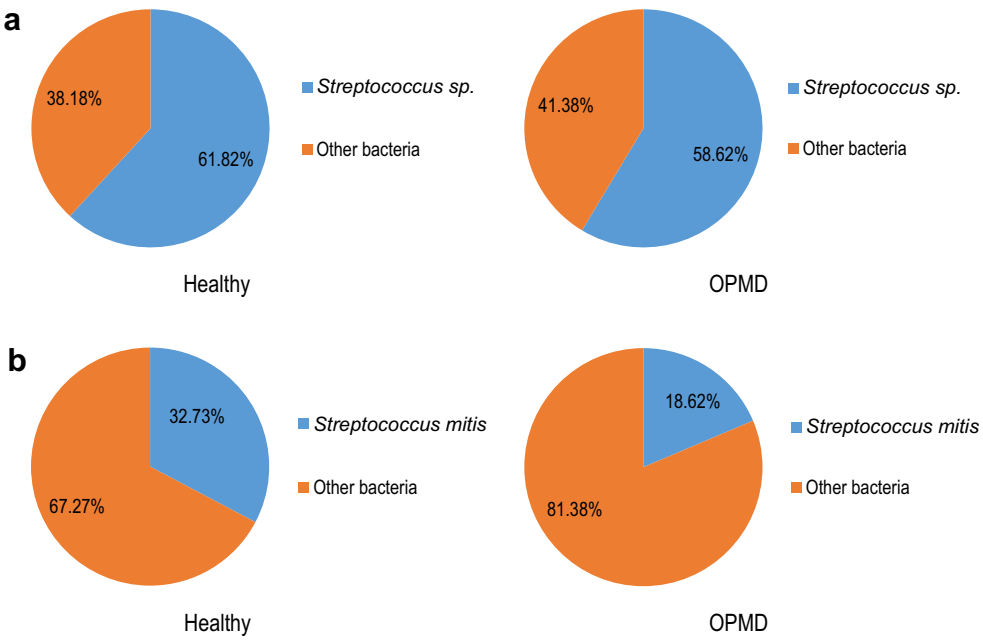

Fig. 5 Ratio of Streptococci in the mucosal lesion a The ratio of Streptococci to all oral bacteria was not significantly different in the healthy tissue compared to the OPMD lesion. **b** The ratio of *Streptococcus mitis* dramatically decreased in the OPMD, as compared to the healthy tissue

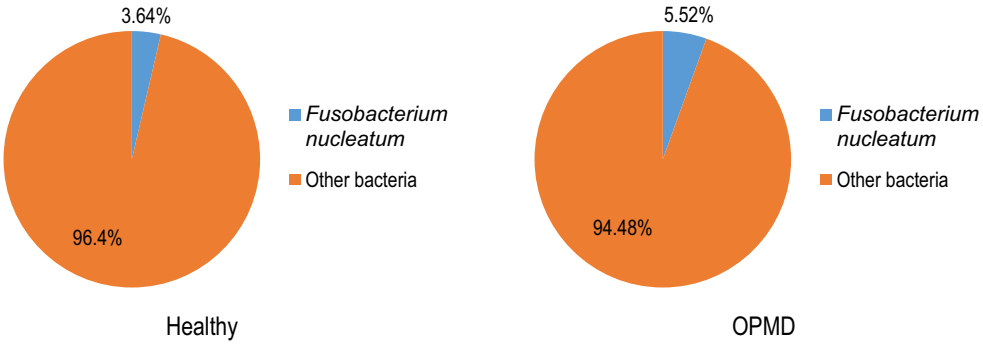

Fig. 6 ***Fusobacterium nucleatum*** **in the mucosal lesion** The ratio of *Fusobacterium nucleatum* was markedly increased in the OPMD lesions, as compared to the healthy tissue detected by metagenome sequencing

a much wider variety of patients are affected [1, 3]. Accordingly, we included a wide range of patients in our sample, without strict limitations on age or sex.

In our study we compared the microbiome and microbiota of healthy and the OPMD tissues of oral cavity.

The association of *Fusobacterium nucleatum* with oral carcinoma was documented [38, 39]. Moreover, it was suggested that distinct subspecies of *F. nucleatum*, such as *F. nucleatum subsp. polymorphum* and *F. nucleatum subsp. vincentii*, may play an etiopathogenetic role in oral carcinogenesis [40]. A study also described the presence of *F. nucleatum* in desquamative gingivitis [41]. However, our report shows the first time the presence of *Fusobacterium nucleatum* in lesion biopsies of OPMD patients.

The finding that *F. nucleatum* is present in OPMD may be exploited to develop a novel therapeutic strategy of distinct oral disorders such as oral lichen planus. In addition, one may speculate that a targeted antibiotic therapy could be beneficial in preventing the development of oral cancer in a subset of OPMD patients.

We observed that the relative abundance of *Streptococcus mitis* decreased dramatically in the pathological niche. Since Streptococci 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 [42–44].

Therefore, it is an interesting and important finding that the relative abundance of *S. mitis* in the OPMD lesions decreased to nearly the half of the healthy area. This observation may possibly be exploited for therapeutic purposes: similarly to the reconditioning of vaginal *Lactobacillus* balance [45], the restitution of *S. mitis* niche in OPMD could also have beneficial effects [46].

Taken together, we presented evidence for the alteration of microbiome and microbiota of OPMD patients. We detected an increased bacterial diversity in the OPMD lesions compared to the healthy oral mucosa. In addition, 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.

Although our study is not suitable to answer the "Chicken or the Egg" problem in all aspects, but we have found that the bacterial colonization of mucosa has already altered in OPMD tissues. These observations may form the basis of novel therapeutic approaches preventing oral carcinogenesis in a subset of patients with OPMD.

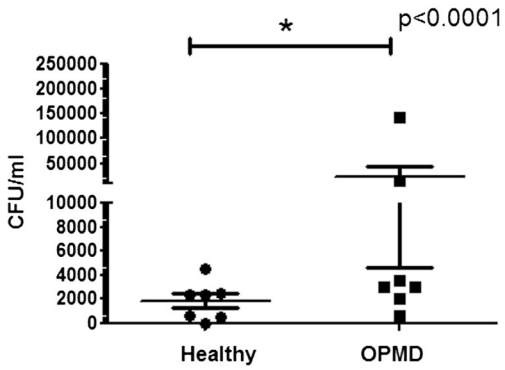

Fig. 7 ***Fusobacterium nucleatum*****-specific PCR of the healthy tissue and the OPMD** OPMD showed significantly higher colonization by *F. nucleatum* compared to healthy mucosa

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Compliance with Ethical Standards

Conflict of Interest No potential conflict of interest was reported by the authors.

Ethical Approval The study protocol conformed to the Declaration of Helsinki in all respects and was approved by the Institutional Ethics Committee of the University of Szeged (No. 3161).

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