MASARYK UNIVERSITY
FACULTY OF MEDICINE

HABILITATION THESIS

Brno 2019

RNDr. Petra Bořilová Linhartová, Ph.D.

MASARYK UNIVERSITY
FACULTY OF MEDICINE
CLINIC OF STOMATOLOGY

IMMUNOGENETICS
OF COMPLEX ORAL DISEASES

Habilitation thesis in the field of Medical Biology

Author:

RNDr. Petra Bořilová Linhartová, Ph.D.

Brno 2019

Abstract

Background and Aim. Host susceptibility to the onset and development of the most common complex oral diseases has been intensively studied in different populations. Candidate genes are selected based on the results from genetic association studies and etiopathogenetic hypothesis of the specific disease affecting the oral cavity. All genes related to both innate and adaptive immunity are the subject of interest in immunogenetics. The main aim of our research was to determine the risk or protective variants of selected genes encoding immunoregulatory factors in relation to recurrent aphthous stomatitis (RAS), external apical root resorption (EARR), dental caries, and chronic periodontitis (CP).

Methods. The Habilitation thesis is a set of commentaries to 10 articles (first author/shared first author in 6/1 cases) published in international journals indexed in the Web of Science database. There are four main parts of the thesis which correspond to one of the selected complex oral diseases: RAS, EARR after orthodontic treatment, dental caries, and CP. The genetic studies were all designed as an association case-control study. Gene variants in interleukins (*IL-1* and its receptor antagonist *IL-1RN*, *IL-2*, *IL-4* and its receptor alpha *IL-4Ra*, *IL-6* and its receptor *IL-6R*, *IL-8* and its receptor *CXCR2*, *IL-10*, and *IL-17*), NOD-like receptor (*NLRP3*), angiotensin converting enzyme (*ACE*), and vitamin D receptor (*VDR*) were determined. Laboratory analyses were based on polymerase chain reaction (PCR) and some studies comprised the implementation of Luminex or enzyme-linked immunosorbent assay (ELISA) techniques, isolation and cultivation of peripheral blood mononuclear cells (PBMCs) with specific stimulants (among others periodontal bacteria) and the molecular detection of oral bacteria.

Results. Variability in the *IL-10* gene and *NLRP3* may play an important role in the development of RAS, especially some *IL-10* haplotypes with proven functional emergence ($P<0.05$). Specific *P2RX7* haplotype predisposing its carrier to increased pro-inflammatory IL-1β secretion was associated with the risk of EARR development ($P<0.05$). *ACE* I/D polymorphism may be associated with caries in permanent ($P<0.05$) but not primary dentition ($P>0.05$), especially in Czech girls. Although the *VDR Taq*I variant cannot be a marker for dental caries risk in our population ($P>0.05$), an association with gingivitis in children was found ($P<0.05$). Significant differences in the allele frequencies of *IL-1B* variant were observed between the controls and CP patients ($P<0.05$) and the specific *IL-1* gene cluster haplotype decreased the risk of CP development ($P<0.01$). Significantly higher

IL-1β levels in PBMCs after stimulation by selected periodontal bacteria, in comparison to unstimulated cells, were found ($P<0.05$). Independently on studied *IL-8* and *CXCR2* polymorphisms with functional emergence, diabetics with CP had higher IL-8 plasma levels than systemically healthy controls with or without CP ($P<0.05$ or $P<0.01$, respectively). In addtion, two *CXCR2* gene variants were associated with subgingival colonization of specific periodontal bacteria in men with CP ($P<0.05$) and *IL-17A* gene variability may influence the "red complex" bacteria occurrence in patients with CP ($P<0.05$).

Conclusions. Some associations between specific gene variants and complex oral diseases were found. Our statistically non-significant findings were also published for reducing the positive bias in the scientific literature. Further studies should unconditionally include not only gene variants determination but also gene expression and protein analysis, together with microbiome research. There is a need to create an algorithm involving all known variables to determine the risk of complex oral disease development in an individual.

Keywords case-control study, gene variant, cytokine, interleukin, aphthous stomatitis, root resorption, dental caries, periodontitis

Conflict of interest

I declare that I have no conflict of interest.

February 21st, 2019 ………………………………………

Author's signature

Acknowledgement

I would like to thank prof. MUDr. Lydie Izakovičová Hollá, Ph.D. for long-term cooperation and critical review of the Habilitation thesis, and to my husband and my parents for their support.

Special thanks belongs to Zachary Kendall from the Charles University, Praha, Czech Republic for linguistic proof.

Funding

The research was supported by:

Ministry of Health of the Czech Republic

- grant NV17-30439A ”Advanced biotechnological and behavioral approaches in dental caries research and prevention strategies”
- grant NV15-29336A ”Changes in immunological reactivity in patients with recurrent aphthas”
- GAČR – grant GB14-37368G ”Centre of orofacial development and regeneration”
- IGA – grant NT11405-6 ”Microbiologic and genetic determinants of the onset and progression of periodontitis in type 1 and 2 diabetics and their reciprocal relationship to diabetes compensation”

and Grant Agency of Masaryk University (MU)

- funds provided by the Faculty of Medicine MU to junior researcher Petra Borilova Linhartova in the years 2016 – 2018
- projects of Specific research

Content

Abstract 3
Conflict of interest..5
Acknowledgement...6
Funding 6
Background and Aim 8
1. Recurrent aphthous stomatitis (RAS) 11
1.1 Comment to the article “Recurrent aphthous stomatitis and gene variability in selected interleukins: a case-control study” 18
1.2 Comment to the article “Association study of interleukin-1 family, interleukin-6, and its receptor gene polymorphisms in patients with recurrent aphthous stomatitis” 37
1.3 Comment to the article “Association of the NOD-like receptor 3 (*NLRP3*) gene variability with recurrent aphthous stomatitis in the Czech population”................................ 45
2. External apical root resorption (EARR) 53
2.1 Comment to the article “Genetic determinants and postorthodontic external apical root resorption in Czech children”... 54
3. Dental caries 64
3.1 Comment to the article “*ACE* insertion/deletion polymorphism associated with caries in permanent but not primary dentition in Czech children” 70
3.2 Comment to the article ”Vitamin D receptor *Taq*I gene polymorphism and dental caries in Czech children” 80
4. Periodontitis...87
4.1 Comment to the article ”Interleukin-1 gene variability and plasma levels in Czech patients with chronic periodontitis and diabetes mellitus”... 90
4.2 Comment to the article ”Differences in interleukin-8 plasma levels between diabetic patients and healthy individuals independently on their periodontal status”......................... 102
4.3 Comment to the article ”Chemokine receptor 2 (*CXCR2*) gene variants and their association with periodontal bacteria in patients with chronic periodontitis”....................... 121
4.4 Comment to the article ”Interleukin-17A gene variability in patients with type 1 diabetes mellitus and chronic periodontitis: its correlation with IL-17 levels and the occurrence of periodontopathic bacteria”.. 131
Conclusions 142
References...144
Figures...157
Attached files 158

Background and Aim

Oral diseases are connected with the disruption of the physiological function of the immune defense mechanisms in the oral cavity. In this context, host saliva, gingival crevicular fluid (GCF), lymphoid tissue (tonsils) and systemic health/disease play crucial roles. Saliva and GCF contain immunoglobulins (secretory IgA), cells of the immune system (lymfocytes, plasmatic cells) with signaling molecules and antimicrobial proteins with non-specific immune responses. The quality and quantity of both fluids and the total immune status are influenced by many factors, among others by host genetic predispositions. With impaired immune defense the dysbiosis of oral microflora is associated, which is reciprocaly related to pathogenic and inflammatory processes in the oral cavity.

Immunogenetics is a breakthrough in immunology and genetics. It deals with the study of the genetic condition of the components of the immune system and the genetic regulation of immune reactions.

The main aim of my research is to determine the risk or protective variants of selected genes encoding immunoregulatory factors in relation to selected complex oral diseases. Complex diseases do not obey the standard Mendelian patterns of inheritance, as their phenotype includes a combination of genetic, environmental, and lifestyle factors (Craig, 2008). The immunogenetic research is important for identifying the hereditary component of a disease associated with immunopathology and may lead to a better understanding of the etiopathogenesis and improved prevention, diagnosis and treatment of the affected patients (Chapple et al., 2017).

An association of polymorphisms with disease reflects the hereditary component of the disease. This means that there are differences in the occurrence of gene variants between patients and the healthy population. If a particular gene variant occurs more frequently among patients than in the general population, it is simply referred to as a variation of higher susceptibility or as a risk variant. In contrast, gene variants which are more rare in patients than in the general population are called protective. The power of association between gene variant and disease can be expressed by general epidemiological parameters such as relative risk or odds ratio (OR). In general, variants for which the OR for a given disease is >1 are risk; protective variants have an OR<1.

Depending on the relationship between a particular polymorphism and a disease, it may be possible to distinguish the direct association where the susceptibility to the disease is caused

by the directly tested gene variant, or indirectly when the tested polymorphism is in a binding imbalance with the causal variant but is not related to the disease itself.

For complex traits, association signals tend to be spread across most of the genome-including many genes without a clear connection to disease. Boyle et al. (2017) reported the hypothesis called the "omnigenic" model. Gene regulatory networks are sufficiently interconnected that all genes expressed in disease-relevant cells are liable to affect the functions of core disease-related genes and that most heritability can be explained by effects on genes outside the core pathways.

The study of candidate genes, usually designed as a case-control association study, is an effective tool for studying the genetic makeup of complex traits. It is methodologically appropriate to include only subjects with extreme phenotypes in the research, for example, groups of subjects with intact dentition (controls) and subjects with severe and multiple dental caries ("cases"). Major gene variability in the number of repetitive gene sequences and single-nucleotide polymorphisms (SNPs) are predominantly observed. Selection of a gene variant it is based on several pieces of available information: functional relevance of the gene and its particular variant, minority allele frequency or repeat rate which should be greater than 5% in the given population, haplotype blocks taken into account, etc. It is known that individual polymorphisms do not exist in isolation, but are genetically linked, forming distinct haplotypes. Haplotype analyses may be of a higher informative value for drawing associations between phenotypes and genetic variation than individual variants.

In contrast to this approach there is a genome-wide association study (GWAS), which identifies SNPs and other variants in DNA associated with a disease, but causal effects of the disease cannot be specified.

Our previous research of complex oral diseases was summarized in my Dissertation thesis (Bořilová Linhartová, 2014). A relationship between some polymorphisms in the genes encoding immunoregulatory factors and external apical root resorption (EARR) after orthodontic treatment or periodontitis in the Czech population was found. Firstly, *IL-1RN**2 allele was found as a risk for EARR in girls. The effect of *IL-4* SNPs on the production of interferon-γ, IL-10, IL-1β a IL-1α, tumor necrosis factor-α a IL-6 after the stimulation of heat shock protein 70 or selected periodontal bacteria was observed in patients suffering from chronic periodontitis (CP). Some *IL-8* haplotypes seemed to be protective for CP and also some *IL-8* SNPs were associated with the occurrence of specific bacteria in controls with healthy gingiva, as well as patients with aggressive periodontitis or CP. In addition,

IL-1 gene cluster variants were associated in our pilot study with CP, or with type 1 diabetes mellitus (T1DM) and with T1DM+CP.

This Habilitation thesis logically and methodically follows our previous research in the Czech population. The chapter about recurrent aphthous stomatitis has been added. Only immunogenetical studies have been included in this work to give focus. Neverthless, our team is works on investigation of oral diseases from a complex point of view, employing *in vitro* experiments, microbiological and biochemical analysis, clinical and pharmacogenetic studies, diseases prevention and behavioural intervention.

1. Recurrent aphthous stomatitis (RAS)

Recurrent aphthous stomatitis (RAS), one of the most common diseases of the oral mucosa, is characterized by the formation of painful oral erosions or even ulcers. RAS diagnosis is based on anamnestical data and appearance of lesions; no laboratory tests to confirm the diagnosis are available.

RAS may appear as an isolated symptom or it may represent an element of systemic syndromes, such as Behcet's disease (BD) or periodic fever with aphthae pharyngitis and adenitis (PFAPA syndrome). It often accompanies gastrointestinal diseases (Mantegazza et al., 2016) and immune dysfunction (Mays et al., 2012).

The disease etiopathogenesis is unknown, but risk factors associated with the origin and development of the disease have been described in the literature. Besides local trauma, food allergens, oral microbial dysbiosis, infectious agents, nutritious factors (deficiency of B_{12} vitamin, iron, and folic acid), stress and hormonal changes, the immunological profile and host genetic predispositions to this multifactorial disease play a role (Fig. 1).

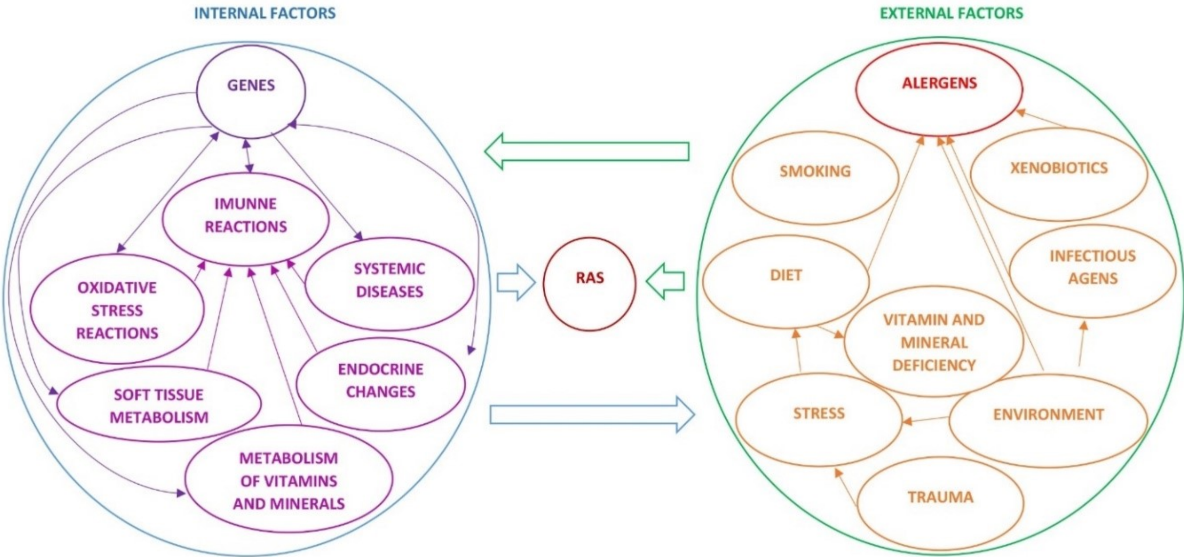


Fig. 1. Interactions between internal and external factors in the etiopathogenesis of recurrent aphthous stomatitis (RAS) (Bořilová Linhartová et al., 2017).

The immunopathogenesis of RAS probably involves a cell-mediated immune response mechanism involving the production of T-cells, ILs and tumor necrosis factor alpha (TNFα). In addition, lymphocyte-mediated mechanisms have been proposed to immune complexes (Jurge et al., 2006). Immune alterations have been observed, beginning with an unknown antigenic stimulation of the keratinocytes and resulting in the activation of T lymphocytes,

cytokine secretion, and leukocyte chemotaxis. Changes have also been reported in elements of the salivary defense system such as the enzyme superoxide dismutase (SOD).

The effect of heredity on the disease origin and development was previously confirmed by studies of twins and families. Genetic variability of the selected genes in patients with RAS compared with healthy controls (case-control study) conducted in different populations have been published. The main candidates for RAS are the genes associated with the immune system, response of the organism to oxidative stress, metabolism of mucosal tissues, vitamins, and minerals.

In our review (Bořilová Linhartová et al., 2017, Attachment 1), findings of genetic association studies were summarized and discussed. Variants in genes encoding interleukin-1 (IL-1) and its receptor antagonist IL-1RA, IL-4, IL-6, IL-10, TNFα, NOD-like receptor 3 (NLRP3), Toll-like receptor 4 (TLR4), E- and L-selectin (SEL), angiotensin converting enzym (ACE), gene for Mediterranean fever (MEFV), serotonin transporter (SLC6A4), matrix metalloproteinase 9 (MMP9), methylenetetrahydrofolate reductase (MTHFR), and nitric oxide syntase 2 (NOS2) were previously studied as factors potentialy influenced the individual susceptibility to the disease development (Fig. 2).

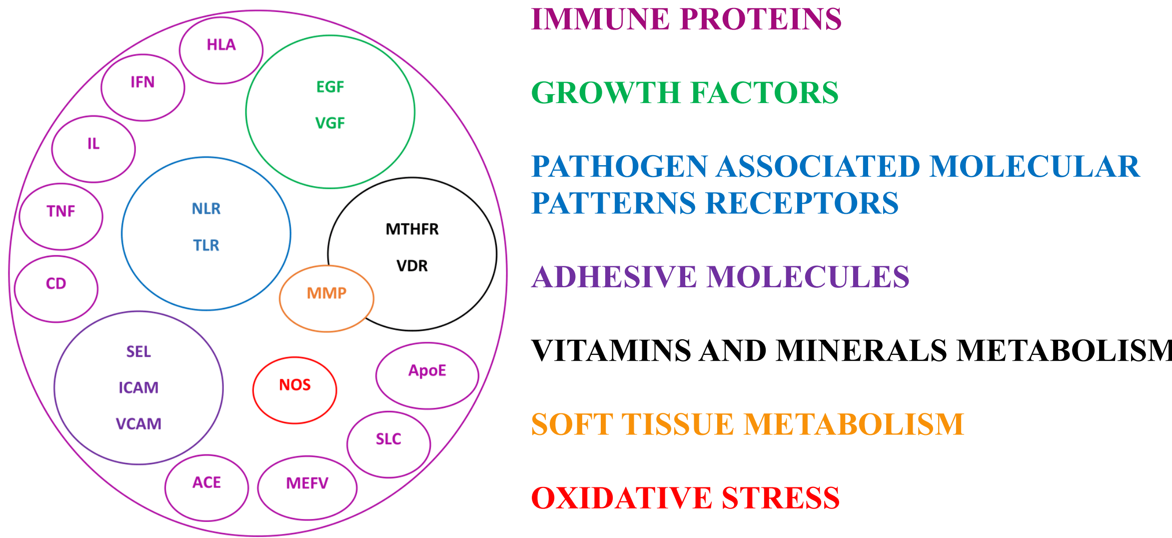


Fig. 2. An overview of proteins or groups of proteins encoded by genes that are considered as candidate for recurrent aphthous stomatitis (RAS) and whose variability has been previously studied (Bořilová Linhartová et al., 2017).

In the context of RAS, genes for ILs with different properties and with possible contradictory effect were previously studied (Fig. 3). We supposed that determining of

immunopathogenetic mechanisms and genetic components can identify and characterize the main factors related to the development of RAS.

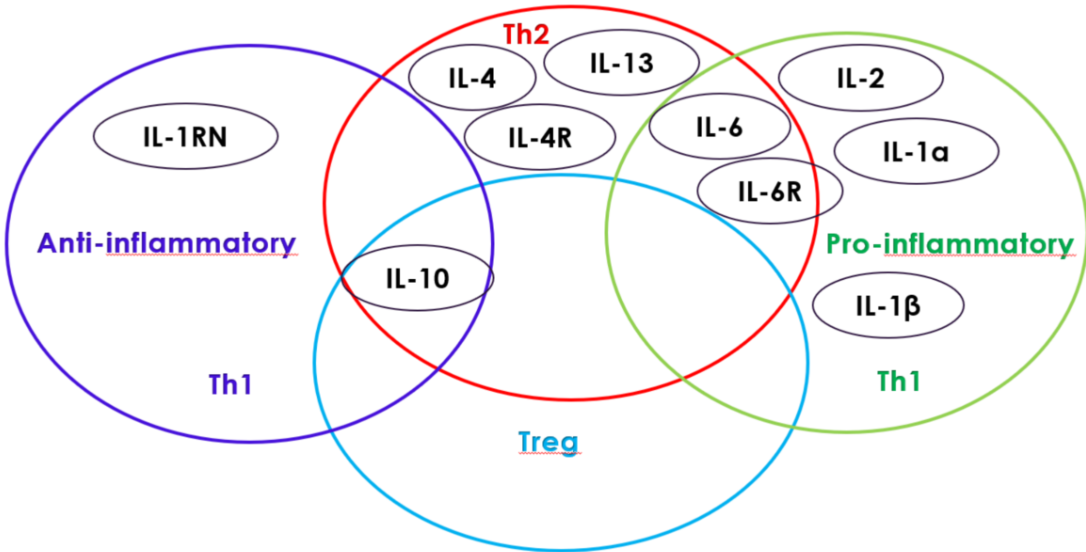


Fig. 3. Classification of selected interleukins (Krejsek et al., 2016) previously studied in context of genetic predispositions to RAS.

Treatment of RAS, drug therapy or laser therapy (Landová et al., 2013; Han et al., 2016), is symptomatic and can reduce pain, frequency incidence or severity of lesions.

So-called Skach's therapy (Škach et al., 1975), in which patients are administered folic acid and vitamin B_6, is often recommended to patients with RAS. Nevertheless, the human body can process folic acid only in the form of active folate (vitamin B_9) and not all people are able to metabolize folic acid effectively. MTHFR is an enzyme that plays an important role in metabolic transformation of folic acid to active folate, and its function is fundamentally influenced by gene polymorphisms. What more, vitamin D deficiency is often observed in patients with both RAS and BD (Ślebioda et al., 2016). Vitamin D_3 (cholecalciferol) is a major immunoregulator, as it has both anti-infective and immunopotentiation as well as immunosuppressive effects. Its supplementation seems to have the potential for both the treatment and the prophylaxis of these conditions (Anand et al., 2017).

In our pilot study (Bořilová Linhartová et al., 2019, Attachment 2), the active form of folic acid together with vitamins B_6 and D_3 were administrated in the three-phase regimen to patients with RAS. Ten patients with genetically impaired ability to metabolize folic acid (poor metaboliser – PM or intermediate metaboliser – IM) and the absence of systemic

disease (Crohn's disease, BD, kidney disease and other diseases) were included. Genomic DNA from these patients suffered by RAS were analyzed for haplogenotypes composed from *MTHFR* rs1801133 and rs1801131 variants. According to the available literature, the predicted phenotype of the patient was determined corresponding to its *MTHFR* haplogenotypic profile. Subjects with 30-65% MTHFR activity are considered IM and patients with lower enzyme function (<30% normal enzyme activity) were identified as PM. Haplogenotype approach was also used in our other pharmacogenetic pilot study focused on proton pump inhibitors metabolism in patients with gastroesophageal reflux disease (Bořilová Linhartová et al., 2018, Attachment 3).
Pharmacotherapy in a modified double-crossed blind study (cross-over design) ran from spring 2018 and lasted three months. Patients were treated with active folate (glucosamine salt 5-methylfolate), vitamins B_6 and D_3, they were not allowed to use any other food supplements during the therapy. They kept daily records of their health status and possible risk factors for RAS and were examined clinically and in the laboratory at monthly intervals. Both the subjective evaluation of the effect of the therapy by patients and the obtained laboratory data were recorded.
The study was comprised of three men and seven women with RAS, aged 28 to 47 years; according to the *MTHFR* gene profile, three were defined as PM and seven as IM of folic acid (Fig. 4).
Based on the subjective assessment, one patient reported a deterioration, two patients did not observe a change in their health state during therapy, and seven were satisfied because they had a lower or no aphthae eruption and/or healing time was significantly shorter (Fig. 5). Subjective evaluation of therapy positively correlated with objective clinical finding ($r=0.993$, $P<0.001$).
The laboratory analysis showed that administration of vitamin D_3 significantly increased serum concentrations of 25-OH D_3 from low/limit to optimal values, median of concentrations prior to study was 54.2 $nmolL^{-1}$, just before administration 68.1 $nmolL^{-1}$ vs. month after administration 96.6 $nmolL^{-1}$ (norm: 50-175 $nmolL^{-1}$), ($P\leq0.01$, Fig. 6).
The other studied parameters, such as concentration of folic acid in serum, homocysteine, ALT, AST, and blood count, were within the physiological range before and after therapy.

SNP	*MTHFR* rs1801131 (A1298C) MAF (C)=34%			
	genotype	AA	AC	CC
	MTHFR enzyme function	100%	80-100%	60%
	EUR population frequencies	43%	45%	12%
MTHFR rs1801133 (C677T), MAF (T)=31%	CC 100% 46%	--/- 100% EM	--/+- 80% EM	--/++ 60% IM
	CT 65% 44%	+-/-- 65% IM	+-/+- 50% IM	+-/++ 30% PM
haplogenotype MTHFR enzyme function predicted phenotype	TT 20%-30% 10%	++/-- <30% PM	++/+- <30% PM	++/++ <10% PM

Fig. 4. Predicted function of MTHFR enzyme by haplogenotype composed from *MTHFR* rs1801133 and rs1801131 variants. Definition of phenotype of studied patients with RAS and their final subjective evaluation of therapy (Bořilová Linhartová et al., 2019).

blue/black/red person=positive/neutral/negative evaluation of therapy by a subject

Note: Allelic and genotype frequencies according to the NCBI database for the European population.

+=minor allele, -=major allele,

EM=extensive (normal) metaboliser

IM=intermediate metaboliser

PM=poor metaboliser

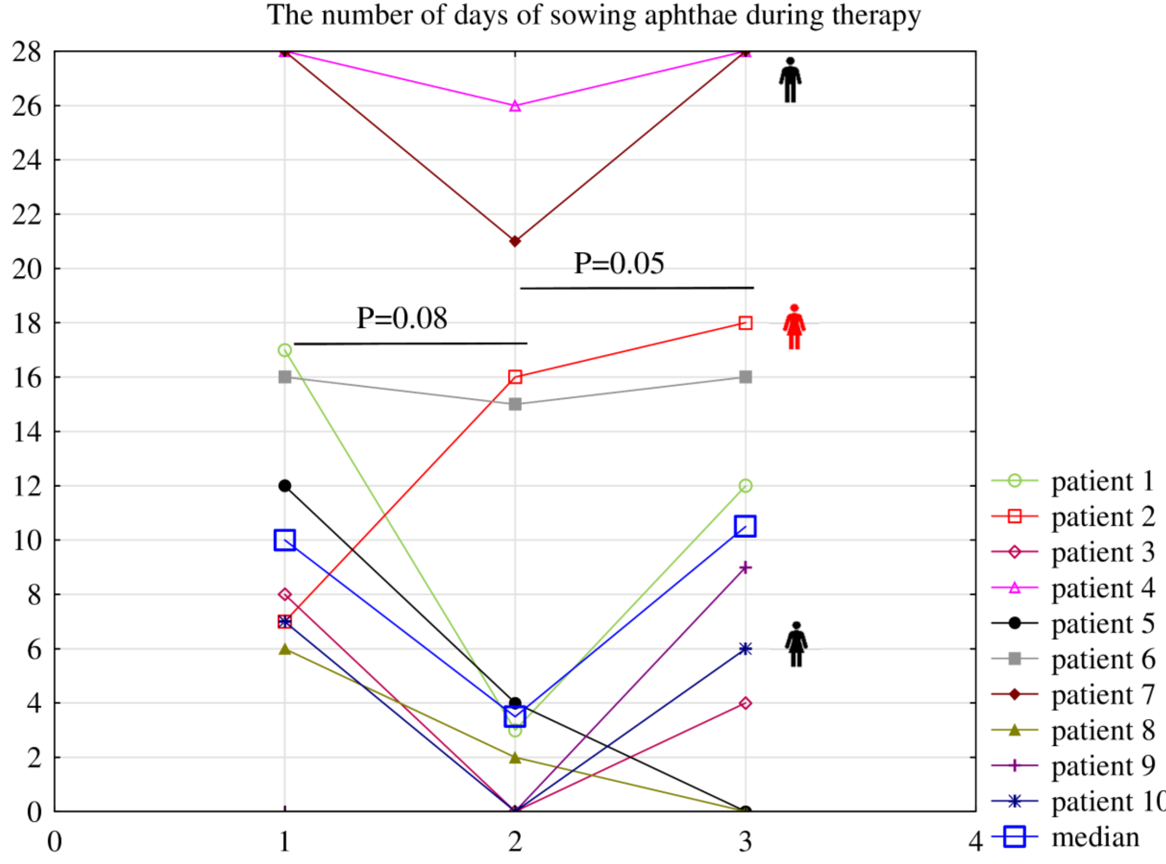


Fig. 5. Number of days of sowing aphthae during therapy (Bořilová Linhartová et al., 2019).

Axis x

1=state during 1st (control) phase

2=status during the 2nd (therapeutic) phase

3=status during the 3rd (tracking) phase

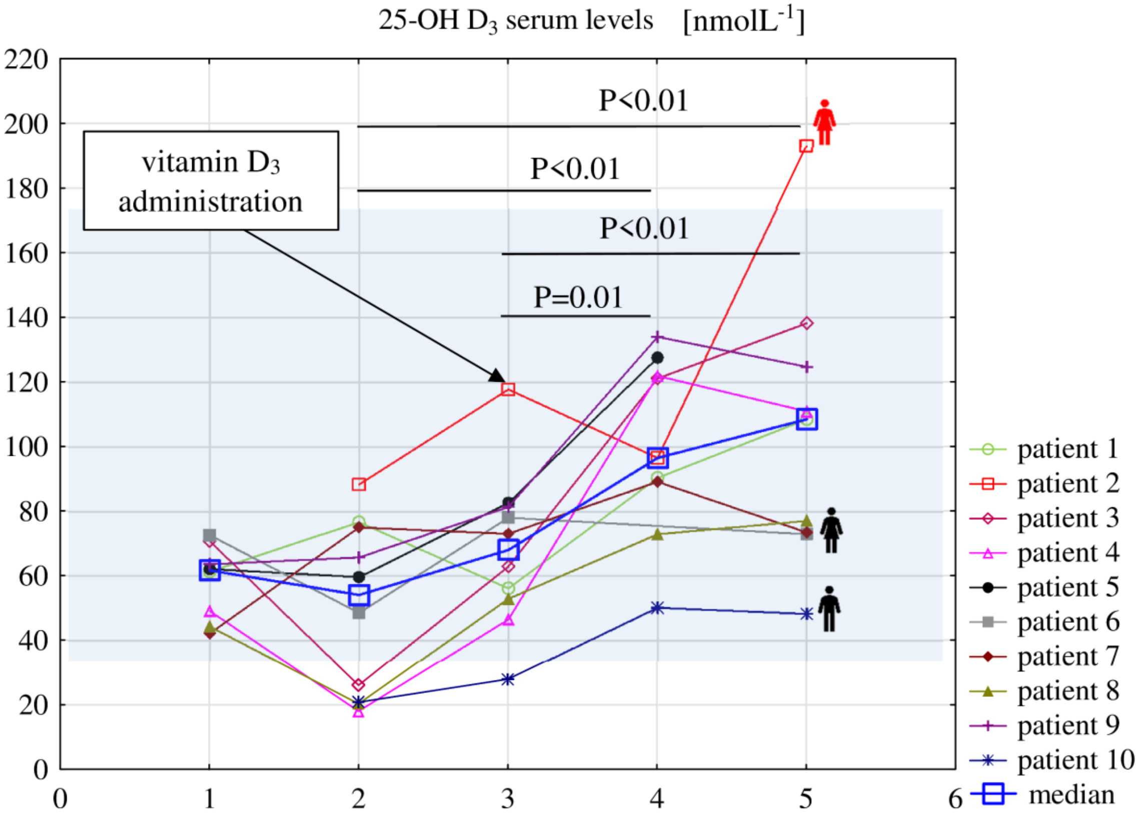


Fig. 6. 25-OH D_3 serum levels in patients suffered by RAS during the therapy (Bořilová Linhartová et al., 2019).

Note: In different patients, four concentration values are missing at different times due to a failure of laboratory analysis.

The blue area shows the serum concentration of 25-OH D_3 (50-175 nmolL-1).

Axis x:

1=status prior to inclusion in the monitored group

2=state before the 1st (control) phase

3=state before the 2nd (therapeutic) phase, after vitamin D_3 administration

4=status after 2nd phase

5=status after 3rd (tracking) phase

The proposed modification of Skach's vitamin therapy seems to be appropriate for the treatment of patients suffering from RAS with genetic predisposition for reduced MTHFR function as during our observation. 70% of patients experienced subjective and objective improvement of their state, so individualized therapy can positively affect the frequency of aphthae/ulcers eruption and the course of their healing in patients with RAS.

1.1 Comment to the article "Recurrent aphthous stomatitis and gene variability in selected interleukins: a case-control study"

Borilova Linhartova P, Janos J, Slezakova S, Bartova J, Petanova J, Kuklinek P, Fassmann A, Dusek L, Izakovicova Holla L. Recurrent aphthous stomatitis and gene variability in selected interleukins: a case-control study. Eur J Oral Sci. 2018;126(6):485-492. DOI: 10.1111/eos.12577. (IF 1.655; DENTISTRY, ORAL SURGERY & MEDICINE Q2)

Author contribution: P.B.L. designed the study, drafted the paper, and carried out genetic analysis.

IL-2 is one of the proinflammatory cytokines produced by Th1 cells. IL-4 and IL-13 are pleiotropic Th2 cytokines produced by various cell types. The functions of IL-13 show considerable overlap with those of IL-4: both proteins exert biological effects via IL-4 receptor alpha (IL-4Ra). IL-10, with broad immunoregulatory functions, is considered as the T-regulatory lymphocyte (Treg or Th3) subset cytokine. IL-2, IL-4, IL-10, and IL-13 are considered as candidate molecules which are involved in the pathogenesis of RAS.

Some of *IL-2*, *IL-4*, and *IL-10* gene polymorphisms were previously associated with RAS in different populations (see Overview in Table S1 on the page 28). To date, *IL-4Ra* and *IL-13* variability has not been studied in any population in the context of RAS.

The aim of our study was: (i) to determine the allele and genotype frequencies of ILs gene polymorphisms in the Czech population sample; (ii) to investigate a possible association of the selected variants and their combination (ie, haplotypes) with susceptibility to RAS; and (iii) to analyze the clinical parameters (oral status) with the individual interleukin genetic profiles. Candidate genes encoding ILs and their specific variants were selected based on associations described previously in other populations, proven functional effects in the etiopathogenesis of RAS, minor allele frequency (MAF) higher than 0.1 in the population studied, and/or localization within the haplotype structure of the gene.

In total, 252 subjects (74 patients with RAS and 178 healthy controls) were enrolled in this case-control study. Polymorphisms *IL-2* rs2069762, *IL-2* rs2069763, *IL-4* rs2243250, *IL-4* rs79071878, IL-4 receptor alpha (*IL-4Ra*) rs1801275, *IL-10* rs1800871, *IL-10* rs1800872, *IL-10* rs1800896, and *IL-13* rs1800925 were determined.

There were no significant differences in the allele or genotype frequencies of the studied *IL-2*, *IL-4*, *IL-4Ra*, *IL-10* (only rs1800896), and *IL-13* polymorphisms between controls and patients with RAS. The minority alleles *IL-10* rs1800871 and *IL-10* rs1800872 were associated with a statistically significant higher risk of RAS, as confirmed by the results of genotype and haplotype analyses. The ATA haplotype composed of *IL-10* (rs1800896, rs1800871, rs1800872) variants was found significantly more frequently in RAS patients than in controls ($P<0.05$, OR=1.63, 95% confidence interval=1.07–2.50), while the ACC haplotype had protective potential in relation to RAS ($P<0.05$, OR=0.58, 95%CI=0.37-0.91). Polymorphisms in the *IL-10* gene may reflect a balance of adaptive responses to autoimmune, infectious and other disease agents (Oleksyk et al., 2009). The GCC haplotype has a higher transcriptional activity when compared with the ATA haplotype, and the carriers of the ATA/ATA haplogenotype produce lower amounts of IL-10 than either of the subjects with other haplogenotypes (Turner et al., 1997; Koss et al., 2000; Reuss et al., 2002; Mangia et al., 2004). In addition, the *IL-10* T allele rs1800871, A allele rs1800872, and T allele rs1554286 were previously associated with BD (Hu et al., 2015).

Variability in the *IL-10* gene may play an important role in the development of RAS in the Czech population. Our results are particularly in line with the findings of Najafi et al. (2014), where the ACC haplotype and CC genotypes for SNPs *IL-10* rs1800871 and *IL-10* rs1800872 were found to be protective for the development of RAS in the Iranian population. However, there are also studies in other populations where no association of *IL-10* gene variants with RAS was confirmed (Bazrafshani et al., 2003; Guimarães et al., 2007; Sun et al., 2013). The differences between these studies may be the result of interpopulation genetic diversity; therefore, further studies in different ethnic groups and larger samples are needed.

European Journal of
Oral Sciences

Eur J Oral Sci 2018; 1–8
DOI: 10.1111/eos.12577
Printed in Singapore. All rights reserved

© 2018 Eur J Oral Sci
European Journal of
Oral Sciences

Recurrent aphthous stomatitis and gene variability in selected interleukins: a case–control study

Petra Borilova Linhartova[1,2], Julius Janos[1], Simona Slezakova[1,2], Jirina Bartova[3], Jitka Petanova[4], Pavel Kuklinek[5], Antonin Fassmann[1], Ladislav Dusek[6], Lydie Izakovicova Holla[1,2]
[1]Clinic of Stomatology, Institution Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Brno; [2]Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno; [3]Institute of Clinical and Experimental Dental Medicine, General University Hospital and First Faculty of Medicine, Charles University, Prague; [4]Department of Immunology and Microbiology, General University Hospital and First Faculty of Medicine, Charles University, Prague; [5]Department of Clinical Immunology and Allergology, Institution Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Brno; [6]Institute of Biostatistics and Analyses, Masaryk University, Brno, Czech Republic

Borilova Linhartova P, Janos J, Slezakova S, Bartova J, Petanova J, Kuklinek P, Fassmann A, Dusek L, Izakovicova Holla L. Recurrent aphthous stomatitis and gene variability in selected interleukins: a case–control study.
Eur J Oral Sci 2018; 00: 1–8. © 2018 Eur J Oral Sci

Genetic factors, especially those related to immune system functioning, have been intensively studied to determine their role in the development of recurrent aphthous stomatitis (RAS). The aim of the present study was to analyze gene variability in interleukin (*IL*)*2*, *IL4* (and its receptor α, *IL4Rα*), *IL10*, and *IL13*, which were selected based on literature review and/or their functional relevance, in Czech patients with RAS and in healthy controls. In total, 252 subjects (178 controls and 74 patients with RAS) were enrolled in this case–control study, and their detailed anamnestic, clinical, and laboratory data were obtained. Nine polymorphisms in the genes encoding interleukins were determined using PCR techniques. There were no significant differences in allele or genotype frequencies of the *IL2*, *IL4*, *IL4Rα*, *IL10*, and *IL13* polymorphisms rs2069762/rs2069763, rs2243250/rs79071878, rs1801275, rs1800896, and rs1800925, respectively, between controls and patients with RAS. The minority alleles rs1800871 and rs1800872, which encode variants of *IL10*, were associated with a statistically significantly higher risk of RAS, as confirmed by the results of genotype and haplotype analyses. We suggest that variability in the *IL10* gene may play an important role in the development of RAS in the Czech population.

Lydie Izakovicova Holla, Clinic of Stomatology and Department of Pathophysiology, Faculty of Medicine, Masaryk University, Pekarska 664/53, 602 00 Brno, Czech Republic

Email: holla@med.muni.cz

Key words: association studies; candidate gene; genetic; immunity; oral ulcer; polymorphisms

Accepted for publication September 2018

Recurrent aphthous stomatitis (RAS) is a common disease of the oral mucosa manifested by aphthous ulceration. The etiopathogenesis of RAS is unknown; however, accumulated data suggest that an abnormal immune reaction might be involved (1). Phagocytic and cytotoxic T-cells probably participate in a destruction of the oral epithelium that is directed and sustained by local cytokine release. Lewkowicz *et al.* (2) suggested that an imbalance in the pro- and anti-inflammatory cytokine network may result in the breakdown of peripheral tolerance in RAS and the heightened immune response toward self-antigens or harmless microorganisms that colonize the oral mucosa.

Risk factors for RAS include genetic predispositions (3), trauma and stress (4, 5), microorganisms, the cessation of smoking, food hypersensitivity, hormonal changes, and vitamin and mineral deficiencies (6, 7). Recurrent aphthous stomatitis may appear as an isolated symptom or it may represent an element of systemic syndromes, such as Behçet's disease (BD) or periodic fever with aphthae pharyngitis and adenitis (PFAPA syndrome). It often accompanies gastrointestinal diseases (8) and immune dysfunction (9).

Heredity is one of the best-defined underlying causes of RAS. Studies on identical twins have shown a hereditary character of this disease, and there is a significantly higher disease concordance in monozygotic twins than in dizygotic twins (10, 11). The familial occurrence is frequent, and about 30%-40% of patients with RAS have another affected family member. Children with RAS-positive parents have a 90% chance of developing RAS and the recurrence of symptoms in these children is more severe (12). Therefore, the inheritance of some specific gene polymorphisms (especially those encoding cytokines) which contribute to formation of aphthous ulcer may predispose family members to RAS (13).

Currently, microarray technologies are used to analyze expression of mRNA for selected genes in recurrent oral ulcer in order to clarify dynamic changes in gene expression (14, 15). To date, variability in the interleukin-1 (*IL1*) gene and its receptor antagonist (*IL1RN*), as well as in *IL2*, *IL4*, *IL6*, *IL10*, *IL12*, and *IL18* have been studied in the context of RAS (see review in Table S1). However, the results of these association studies have often been contradictory in different populations. Positive associations were found between RAS and selected *IL1*, *IL2*, *IL4*, *IL6*, and *IL10* gene polymorphisms. Interestingly, only 4 genetic studies have focused on the gene variability of

interleukins in European Caucasian populations with RAS (16–19), even though the prevalence of this disease is comparable across populations (20–23). As *IL1* and *IL6* gene polymorphisms have already been studied in RAS patients from the Czech Republic (19), the current study deals with *IL2*, *IL4* and its receptor α, *IL4Rα*, *IL10*, and *IL13*. The selection of these genes is also based on the previously described findings of changes in IL-2, IL-4, IL-10, and IL-13 protein/mRNA levels in patients with RAS compared with controls (24–28), which may be a result of the variability in individual genes of patients.

The aim of our study was: (i) to determine the allele and genotype frequencies of interleukin gene polymorphisms in the Czech population sample; (ii) to investigate a possible association of selected variants and their combination (ie. haplotypes) with susceptibility to RAS; and (iii) to analyze clinical parameters (oral status) with the individual interleukin genetic profiles. Candidate genes encoding interleukins and their specific variants were selected based on associations described previously in other populations (see Table S1), proven functional effects in the etiopathogenesis of RAS, minor allele frequency (MAF) higher than 0.1 in the population studied, and/or localization within the haplotype structure of the gene.

Material and methods

Subjects and clinical examinations

In total, 252 subjects were enrolled in this study: 74 patients with RAS and 178 healthy controls. Patients with RAS were recruited from the Department of Immunology and Microbiology, General University Hospital and First Faculty of Medicine, Charles University, Prague, and from the Department of Clinical Immunology and Allergology, Institution Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Brno. The diagnosis of RAS was made by oral medicine specialists based on accepted clinical criteria (29). Recurrent aphthous stomatitis was divided into 3 clinical subtypes, according to Karakus *et al.*: (i) major (larger than 1 cm in diameter and deeper than the minor form; healing within 10–30 d); (ii) minor (<1 cm in diameter; healing within 10–14 d); and (iii) herpetiform aphthae (grouped aphthae, 1–2 mm in size) (30). Patients were examined by 4 calibrated examiners, according to the same protocol, and detailed anamnestic, clinical, and laboratory data were obtained. The inclusion criteria for cases were willingness to participate and compliance with the diagnostic criteria for RAS. To exclude other disorders (such as celiac disease, BD, Crohn's disease, clinical neutropenia, vitamin B12 deficiency, and erythema multiformae), routine laboratory tests, clinical immunologic investigation, and immunologic tests were performed as described in our previous study (31).

The control group consisted of age- and sex-comparable healthy individuals without a history of RAS or any systemic diseases, and who were selected from general medical practice registers. The inclusion criteria for controls were willingness to participate. The exclusion criteria for both groups were the presence of any other significant systemic diseases (such as cardiovascular disease, diabetes mellitus, gastrointestinal disease, malignancies, and autoimmune diseases). Nursing and pregnant women were also excluded from our study.

Written informed consent was obtained from all participants, in line with the Declaration of Helsinki, before inclusion in the study, which was performed with the approval of the Committees for Ethics of the Faculty of Medicine, Masaryk University Brno (39/2015), St. Anne's Faculty Hospital (8G/2015), and General University Hospital and First Faculty of Medicine, Charles University, Prague (53/2014).

Genetic analysis

Genomic DNA was obtained from peripheral blood leukocytes, according to a standard method, by phenol-chloroform extraction and proteinase K digestion of cells. Isolation, storage of DNA, and genotyping of samples were conducted in the laboratory of the Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.

In total, 9 polymorphisms were analyzed. Five – *IL2* (rs2069762), *IL2* (rs2069763), *IL4* -590C/T (rs2243250), *IL4Rα* A/G (rs1801275, Q576R), and *IL13* -1112 C/T (rs1800925) – were analyzed with PCR using 5′ nuclease TaqMan assays (C__15859930_10, C__15859920_10, C__16176216_10, C___2351160_20, and C___8932056_10, respectively). Real-time PCR protocols were designed according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA), fluorescence was measured using the ABI PRISM 7000 Sequence Detection System (Thermo Fisher Scientific), and SDS version 1.2.3 software (Thermo Fisher Scientific) was used to analyze real-time and endpoint fluorescence data. The remaining 4 polymorphisms were analysed using the variable number of tandem repeat (VNTR) PCR method or by PCR followed by restriction fragment length polymorphism (RFLP-PCR).

The VNTR PCR method was used to detect 70-bp tandem repeat polymorphisms in intron 3 of *IL4* (rs79071878) with slight adaptations (32); details of the genotyping method were published previously (33).

The promoter single-nucleotide polymorphisms (SNPs) *IL10* -1082 A/G (rs1800896), *IL10* -819 C/T (rs1800871), and *IL10* -592 C/A (rs1800872) were determined using the PCR-RFLP method. PCR was carried out in a volume of 45.0 μl containing 250 ng of genomic DNA, 0.35 μM each primer (forward: AAGACAACACTAAGGCTTCCTT; reverse: TAAATATCCTCAAAGTTCC) previously designed (34), 5 U of DNA polymerase (Thermo Fisher Scientific), 4 mM $MgCl_2$, 10× $MgCl_2$-free reaction buffer with 200 mM $(NH_4)_2SO_4$ (Thermo Fisher Scientific), and 0.9 mM deoxyribonucleoside triphosphate mix (Thermo Fisher Scientific). The reaction was carried out in a Sensoquest labcycler (Schoeller Instruments, Praha, Czech Republic). First of all, the samples were denatured by incubation for 5 min at 95°C. Then, samples were amplified over 30 cycles of 30 s at 95°C, 30 s at 45°C, and 72°C for 1 min. This was followed by a final extension of 7 min at 72°C. Then, three 10.0 μl aliqoutes of PCR product were prepared and incubated separately with the restriction enzymes *Eco*NI (13.5 U; New England Biolabs), *Mae*III (1 U; Roche, Basel, Switzerland), or *Rsa*I (1.5 U, New England Biolabs), according to the manufacturer's instructions. After incubation, the contents of each tubes were loaded into separate wells of a 3.0% agarose gel, which contained 10 mg mL^{-1} ethidium bromide and electrophoresed at 90 V. Sizing of products was performed using a 50-bp ladder (Thermo Fisher Scientific). The lengths of the fragments after digestion were as follows: (i) 306 + 278 bp

(GG) or 306 + 253 + 5 bp (AA) after use of *Eco*NI for determination of the *IL10* -1082 A/G (rs1800896) genotype; (ii) 288 + 217 + 79 bp (CC) or 505 + 79 bp (TT) after use of *Mae*III for determination of the *IL10* -819 C/T (rs1800871) genotype; and (iii) 305 + 229 + 42 + 8 bp (CC) or 240 + 229 + 65 + 42 + 8 bp (AA) after use of *Rsa*I for determination of the *IL10* -592 C/A (rs1800872) genotype.

Three researchers carried out the genetic analysis and were unaware of the phenotype; 10% of samples were analyzed in duplicate.

Statistical analysis

Standard descriptive statistics, that is, absolute and relative frequencies for categorical variables and mean and SD for quantitative variables, were applied. The chi-square and Fisher's exact tests were used for comparison of differences in the genotype or allele frequencies among cases and controls. The program SNP Analyzer 2 (available at http://snp.istech21.com/snpanalyzer/2.0/) was used to determine haplotype frequencies. Differences between patient and control groups were tested using a simulation based on the Monte Carlo method. In this test, 10,000 random simulations were performed to generate a set of haplotype distribution tables subsequently tested for the frequency equal to, or more extreme than, the observed data. Empirical probabilities were transformed and tested using the chi-square test. Linkage disequilibrium (LD) and the squared correlation coefficient (r^2) were calculated using Haploview software (Broad Institute, Cambridge, MA, USA). In Fig. 1, black triangles indicate the LD blocks identified by Haploview using the Solid spine of the LD method. Odds ratios (OR) and their 95% CI, together with *P* values, were calculated. All statistical analyses were performed using the program package Statistica v. 13 (StatSoft, Tulsa, OK, USA).

Results

The study group comprised 74 patients with RAS (42 men and 32 women; mean age: 39.9 ± 15.1 yr) and 178 unrelated healthy age- and gender-comparable control subjects (85 men and 93 women; mean age: 47.1 ± 12.2 yr) (Table 1). Most (95.9%) of the patients with RAS had a minor apthae; no subjects had a herpetiform type of this disease.

The final sample size (74 vs. 178) was checked to determine whether it was of sufficient size and quality for the results to have statistically significant power, based on standard assumptions (power: 0.80; α: 0.05). The sample size was sufficient to ensure a statistically relevant detection of difference of 0.15 in relative frequencies based on 2-sample testing. The genotype frequencies of 8 polymorphisms were in Hardy–Weinberg equilibrium (HWE, $P > 0.05$); only the SNP *IL10* (rs1800896) was not in genetic balance in our population (HWE = 0.997, $P < 0.01$).

The distribution of alleles and genotypes of *IL2* (rs2069762 and rs2069763), *IL4* (rs2243250 and rs79071878), *IL4Rα* (rs1801275), *IL10* (rs1800896), and *IL13* (rs1800925) was similar between the control group and the RAS group (Table 2). Furthermore, the haplotype analysis of *IL2* or *IL4* showed no association between their variants and RAS (Tables 3 and 4).

On the other hand, the T allele of SNP *IL10* (rs1800871) and the A allele of SNP *IL10* (rs1800872) were associated with RAS ($P = 0.015$, OR = 1.63, 95% CI = 1.07–2.46; and $P = 0.007$, OR = 1.73, 95% CI = 1.14–2.62, respectively) (Table 2). Furthermore, in the genotype and haplotype analyses, the significant differences between RAS patients and controls were observed in these 2 *IL10* loci. In comparison with the CC+CT genotypes, carriers of the TT genotype of SNP *IL10* (rs1800871) had a higher risk of RAS development ($P = 0.02$, OR = 2.93, 95% CI = 1.19–7.24); similarly, subjects with the AA genotype of SNP *IL10* (rs1800872) are at higher risk for developing RAS than are patients with other genotypes ($P = 0.02$, OR = 2.93, 95% CI = 1.19–7.24). The ATA haplotype composed

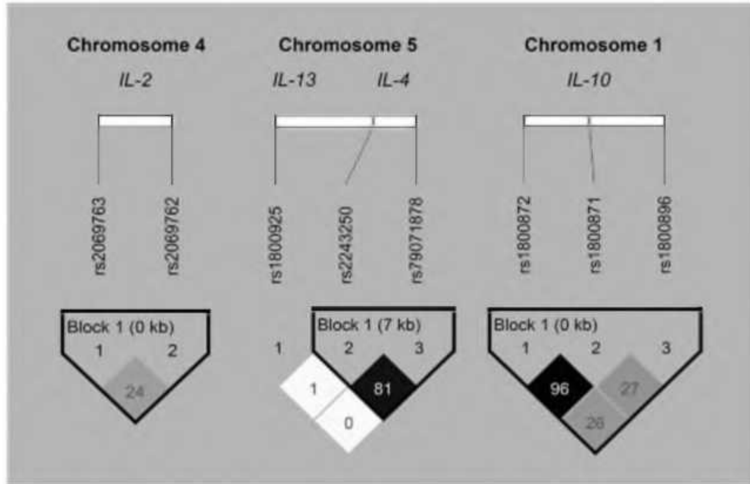


Fig. 1. Patterns of linkage disequilibrium (LD) between interleukin (*IL*)*2*, *IL4* and *IL13*, or *IL10* variants. High pairwise LD [squared correlation coefficient (r^2)] between markers is illustrated with dark shading. The r^2 values (×100) for the marker pairs are listed in the corresponding boxes.

Table 1

Clinical characteristics of the subjects examined in this study

Clinical parameters	Control group	RAS group
Mean age (yr)	47.1 ± 12.2	39.9 ± 15.1
Sex (men/women)	85/93 (47.8/52.2)	42/32 (56.8/43.2)
Type of apthae		
Minor	NA	71 (95.9)
Major	NA	3 (4.1)
Herpetiform	NA	0 (0.0)
Mean number of lesions in each episode		
<3 lesions	NA	26 (35.1)
≥3 lesions	NA	48 (64.9)
Duration of lesions to healing		
<1 wk	NA	36 (48.6)
<2 wk	NA	21 (28.4)
≥2 wk	NA	17 (23.0)
Number of oral ulcer recurrences		
<3 times per yr/irregularly	NA	4/4 (5.4/5.4)
At least 1 per 3 months	NA	16 (21.6)
At least 1 per month	NA	14 (18.9)
At least 2 times per month	NA	12 (16.2)
Permanently	NA	24 (32.4)

Values are given as mean ± SD or *n* (%).
NA, not applicable; RAS, recurrent aphthous stomatitis.

Table 2

*Genotype and MAF of interleukin (*IL*)*2, IL4 *and its receptor (*IL4Rα*),* IL10, *and* IL13 *polymorphisms in the control and recurrent aphthous stomatitis (RAS) groups*

Polymorphism	Genotypes and minor allele	Control group (n = 178)	RAS group (n = 74)
IL2	AA	68 (46.6)	28 (37.8)
-330 A/C	AC	56 (38.3)	34 (46.0)
(rs2069762)*	CC	22 (15.1)	12 (16.2)
	C (C)	34.2	39.2
IL2	CC	67 (45.9)	34 (46.0)
+166 A/C	CA	63 (43.1)	32 (43.2)
(rs2069763)*	AA	16 (11.0)	8 (10.8)
	C (A)	32.5	32.4
IL4	CC	122 (68.5)	46 (62.2)
-590 C/T	CT	50 (28.1)	25 (33.8)
(rs2243250)	TT	6 (3.4)	3 (4.1)
	C (T)	17.4	20.9
IL4	33	123 (69.1)	46 (62.2)
70 bp VNTR	32	51 (28.7)	26 (35.1)
intron 3	22	4 (2.2)	2 (2.7)
(rs79071878)	C (2)	16.6	20.3
IL4Rα	AA	114 (64.0)	52 (70.3)
A/G	AG	57 (32.0)	19 (25.7)
(rs1801275, Q576R)	GG	7 (3.9)	3 (4.0)
	C (G)	19.9	16.9
IL10	AA	50 (28.1)	19 (25.7)
-1082 A/G	AG	101 (56.7)	43 (58.1)
(rs1800896)†	GG	27 (15.2)	12 (16.2)
	C (G)	43.5	45.3
IL10	CC	99 (55.6)	33 (44.6)
-819 C/T	CT	69 (38.8)	30 (40.5)
(rs1800871)	TT	10 (5.6)	11 (14.9)§
	C (T)	25.0	35.1§
IL10	CC	103 (57.9)	33 (44.6)
-592 C/A	CA	65 (36.5)	30 (40.5)
(rs1800872)	AA	10 (5.6)	11 (14.9)§
	C (A)	23.9	35.1§
IL13	CC	91 (53.8)	42 (56.8)
-1112 C/T	CT	68 (40.2)	31 (41.9)
(rs1800925)‡	TT	10 (5.9)	1 (1.4)
	C (T)	26.0	22.3

Values are given as *n* (%) (MAF).
Comparisons were performed using Fisher's exact or chi-square tests.
*Only 146 controls were genotyped.
†Not in Hardy-Weinberg equilibrium.
‡Only 169 controls were genotyped.
§$P < 0.05$.
MAF, minor allele frequency; VNTR, variable number of tandem repeat.

of *IL10* (rs1800896, rs1800871, rs1800872) variants was found significantly more frequently in RAS patients than in controls ($P < 0.05$, OR = 1.63, 95% CI = 1.07–2.50), while the ACC haplotype had protective potential in relation to RAS ($P < 0.05$, OR = 0.58, 95% CI = 0.37–0.91) (Table 5). Pairwise LD for all possible 2-way comparisons among 2 variants in the *IL2* gene, 3 polymorphisms in the *IL4* and *IL13* genes, and 3 polymorphisms in the *IL10* gene cluster in RAS and control groups was determined according to r^2 (see Fig. 1).

Discussion

Research into the role of gene polymorphisms in RAS is ongoing. Among others, cytokines such as IL-2, IL-4, IL-10, and IL-13 may be considered as candidate genes for RAS (35). Defining the RAS etiologic causes among genetic factors may, in future, help determine the risk of disease onset (13).

Increased salivary levels of IL-2, one of the proinflammatory cytokines produced by T-helper (Th)1 cells, were found in patients with RAS compared with healthy controls (36). While no statistical differences were observed in the serum levels of IL-2 between patients with RAS and controls in a Turkish population (37), Sun *et al.* (26) suggested that the markedly increased plasma level of IL-2 may have resulted from secretion by the increased number of activated $CD4^+$ cells and that the expression of IL-2R by activated peripheral blood lymphocytes was upregulated by the plasma level of IL-2 in patients with RAS.

A relationship between *IL2* SNPs/*IL2* haplotypes and RAS development was not found in our Caucasian population. This is in line with the results of a study by Sun *et al.* (38), also showing that MAFs are similar in Chinese healthy subjects and Czech controls. On the other hand, protective and risk *IL2* genotypes were associated with RAS in an Iranian population (39). The differences between these studies may be a result of interpopulation genetic diversity; therefore, further studies in different ethnic groups and larger samples are needed.

Interleukins 4 and 13 – pleiotropic Th2 cytokines – are produced by various cell types. The IL-4/IL-13 axes are most often discussed in relation to asthma, atopic

Table 3

Haplotype frequencies of interleukin-2 (IL2) polymorphisms in control and recurrent aphthous stomatitis (RAS) groups

IL2 -330 A/C (rs2069762)	*IL2* +166 A/C (rs2069763)	Control group *n* (%)	RAS group *n* (%)	OR (95% CI)
A	C	34.2	28.4	0.77 (0.50–1.19)
C	C	33.2	39.2	1.28 (0.85–1.92)
A	A	31.5	32.4	1.03 (0.67–1.57)
C	A	1.0	0.0	0.00 (0.00–0.00)

Haplotypes are ordered according to the decreasing haplotype frequency in healthy controls.
Comparisons were performed using the chi-square test.

Table 4

Haplotype frequencies of interleukin 4 (IL4) polymorphisms in control and recurrent aphthous stomatitis (RAS) groups

IL4 -590 C/T (rs2243250)	*IL4* 70 bp VNTR intron 3 (rs79071878)	Control group *n* (%)	RAS group *n* (%)	OR (95% CI)
C	3	81.1	78.4	0.84 (0.52–1.35)
T	2	15.1	19.6	1.36 (0.83–2.24)
T	3	2.3	1.4	0.60 (0.13–2.84)
C	2	1.4	0.7	0.48 (0.06–4.12)

Haplotypes are ordered according to the decreasing haplotype frequency in the healthy controls.
Comparisons were performed using the chi-square test.
VNTR, variable number of tandem repeat.

dermatitis, allergic rhinitis, chronic obstructive pulmonary disease, cancer, inflammatory bowel disease, autoimmune disease, and fibrotic disease (40). Lewkowicz *et al.* (41) claimed that cells in aphthous ulcers expressed a characteristic Th1-like gene profile, whereas Th2-related genes were not overexpressed in RAS tissues, except for chemokine receptor 3 (CCR3). In another study, higher expression of IL-4, the Th2 cytokine inhibiting Th1 differentiation and activity, was observed in oral ulcers of patients with BD but not in patients with RAS in comparison with healthy controls (24). Serum levels of IL-13 were higher in patients with RAS and in patients with BD than in healthy controls (25).

The functions of IL-13 show considerable overlap with those of IL-4: both proteins exert biological effects via IL-4Rα (gene position 16p12.1-p11.2) and their genes are located in the 5q31.1 region. The *IL13* gene encodes a cytokine that induces immunoglobulin E (IgE) secretion from activated human B cells (42). Interleukin 13 is an important mediator of the physiological changes induced by allergic inflammation.

Also, IgE levels may be considered as part of the RAS patient's work-up (43). The *IL13* polymorphism (rs1800925) influenced IgE expression (44); thus, *IL13* genotypes can play a role in genetic susceptibility to allergy via regulation of serum total IgE levels and by affecting expression of the gene encoding interferon-γ (45). Gene variant *IL4* (rs2243250) has previously been shown to be associated with enhanced IL-4 activity, resulting in higher total serum IgE levels (46).

Although haplotype analyses based on polymorphisms in *IL4* and *IL13* have been published previously (47), in this study, LD/r^2 between *IL13* SNP and 2 polymorphisms in *IL4* were very low; therefore, the haplotype block included only the *IL4* variants (Fig. 1). In contrast to the study by Kalkan *et al.* (48), no association between *IL4* (rs79071878) and RAS was found in a Czech population. The *IL4* (rs79071878) MAF was found 13.6% in European population (49), which is in line with our finding. (49) In addition, frequencies of the SNPs studied in *IL4*, *IL4Rα*, and *IL13* did not differ significantly between RAS patients and controls and, furthermore, the haplotype analysis of *IL4* variants did not reveal any association with RAS (see Tables 2 and 4).

Interleukin-10, with broad immunoregulatory functions, is considered as the T-regulatory lymphocyte (Treg or Th3) subset cytokine. The enhanced production of IL-10 protects against inflammatory diseases, whereas lowered production helps eliminate foreign pathogens by the host (50). The IL-10 levels were found to be lower in patients with RAS than in controls (27, 28). Although no significant increase in the *IL10* mRNA level was recorded in RAS lesions, Buño *et al.* (51) suggested that the failure to suppress the inflammatory reaction initiated by trauma or other external stimuli is likely to involve a functional deficiency of IL-10 in the oral mucosa, which may play a

Table 5

Haplotype frequencies of interleukin 10 (IL10) polymorphisms in the control and recurrent aphthous stomatitis (RAS) groups

IL10 -1082 A/G (rs1800896)	*IL10* -819 C/T (rs1800871)	*IL10* -592 C/A (rs1800872)	Control group *n* (%)	RAS group *n* (%)	OR (95% CI)
G	C	C	43.5	43.5	1.02 (0.69–1.50)
A	C	C	31.5	21.3*	0.58 (0.37–0.91)
A	T	A	23.9	33.4*	1.63 (1.07–2.47)
A	T	C	1.1	0.0	0.00 (0.00–0.00)
G	T	A	0.0	1.7	0.00 (0.00–0.00)

Haplotypes are ordered according to decreasing haplotype frequency in healthy controls.
Comparisons were performed using the chi-square test.
*$P < 0.05$.

role in the pathogenesis of RAS. Interestingly, the numbers of T cells secreting IL-4 were found to be decreased (opposite to IL-10 which was increased) in patients with RAS compared with controls (52). Because IL-10 stimulates epithelial proliferation in the healing stage, expression of a low level of IL-10 may indicate prolonged duration of the ulcer (53).

The study by Oleksyk *et al.* (50) reached the conclusion that polymorphisms in *IL10* may reflect a balance of the adaptive responses to autoimmune, infectious, and other disease agents. The T (rs1800871), A (rs1800872), and T (rs1554286) alleles in *IL10* have previously been associated with BD (54). The GCC haplotype has a higher transcriptional activity than the ATA haplotype, and the carriers of the ATA/ATA haplogenotype produce lower amounts of IL-10 than either of the subjects with other haplogenotypes (55–58).

In the present study, variants of SNPs *IL10* (rs1800871) and *IL10* (rs1800872) were associated with RAS. In addition, the *IL10* ATA haplotype was determined as a risk factor for RAS development (33.4% in RAS patients vs. 23.9% in controls), while the ACC haplotype was found with a lower frequency in the RAS group than in the control group (21.3% vs. 31.5%). Our results are particularly in line with findings of Najafi *et al.* (59), in which the ACC haplotype and CC genotypes for SNPs *IL10* (rs1800871) and *IL10* (rs1800872) were found to be protective for the development of RAS in an Iranian population. However, there are also studies in other populations in which no association of *IL10* gene variants with RAS was found (Table S1) (17, 38, 60).

There are certain limitations of this study. The study population included only 74 patients with RAS. However, the number of patients from this Czech population is comparable with the size of cohorts in other studies; see Table S1 (38, 39, 59, 60). Another drawback of this study is that the levels of IL-2, IL-4, IL-4Rα, IL-10, and IL-13 were not measured; however, the functional relevance of the gene variants selected for the analysis is mostly known.

On the other hand, gene variability in selected interleukin genes (except for 2 *IL10* gene variants) (17) in relation to RAS have not previously been analyzed in European Caucasian populations. To date, *IL4Rα* and *IL13* variability has not been studied in any population in the context of RAS. The data presented from the analysis of interleukin proteins indicate that *IL2*, *IL10*, and *IL13* may be involved in RAS development and thus they may be considered as candidate genes for this oral disease. Nevertheless, no effect of the *IL2*, *IL4*, *IL4Rα*, or *IL13* SNPs themselves on the development of RAS was found in this Czech population. In conclusion, we suggest that the variability in *IL10* may be an important factor contributing to the etiopathogenesis of RAS in European Caucasian populations.

Acknowledgements – The study was supported by the Ministry of Health of the Czech Republic, grants NV15-29336A and GACR GB14-37368G, funds provided by the Faculty of Medicine MU to junior researcher Petra Borilova Linhartova, and the project MUNI/A/1008/2017. *Author contributions:* PBL, JB, JP, and LIH designed the study and drafted the paper; JB, JP, PK, and AF performed the clinical analysis and collected the blood samples; PBL, JJ, and SS carried out the genetic analysis; and LIH and LD performed the statistical analysis. All authors revised the final version of the manuscript.

Conflict of interest – The authors confirm that they have no conflict of interests to declare.

References

1. Laskaris G. Aphthous stomatitis. In: Katsambas AD, Lotti TM, Dessinioti C, D'Erme AM, eds. *European handbook of dermatological treatments.* Berlin, Heidelberg: Springer, 2015: 67–71.
2. Lewkowicz N, Lewkowicz P, Banasik M, Kurnatowska A, Tchorzewski H. Predominance of Type 1 cytokines and decreased number of CD4(+)CD25(+high) T regulatory cells in peripheral blood of patients with recurrent aphthous ulcerations. *Immunol Lett* 2005; **99**: 57–62.
3. Koybasi S, Parlak AH, Serin E, Yilmaz F, Serin D. Recurrent aphthous stomatitis: investigation of possible etiologic factors. *Am J Otolaryngol* 2006; **27**: 229–232.
4. De Barros Gallo C, Mimura MA, Sugaya NN. Psychological stress and recurrent aphthous stomatitis. *Clinics (Sao Paulo)* 2009; **64**: 645–648.
5. Gavic L, Cigic L, Biocina Lukenda D, Gruden V, Gruden Pokupec JS. The role of anxiety, depression, and psychological stress on the clinical status of recurrent aphthous stomatitis and oral lichen planus. *J Oral Pathol Med* 2014; **43**: 410–417.
6. Sun A, Chen HM, Cheng SJ, Wang YP, Chang JY, Wu YC, Chiang CP. Significant association of deficiencies of hemoglobin, iron, vitamin B12, and folic acid and high homocysteine level with recurrent aphthous stomatitis. *J Oral Pathol Med* 2015; **44**: 300–305.
7. Ozler GS. Zinc deficiency in patients with recurrent aphthous stomatitis: a pilot study. *J Laryngol Otol* 2014; **128**: 531–533.
8. Mantegazza C, Paglia M, Angiero F, Crippa R. Oral manifestations of gastrointestinal diseases in children. Part 4: Coeliac disease. *Eur J Paediatr Dent* 2016; **17**: 332–334.
9. Mays JW, Sarmadi M, Moutsopoulos NM. Oral manifestations of systemic autoimmune and inflammatory diseases: diagnosis and clinical management. *J Evid Based Dent Pract* 2012; **12**: 265–282.
10. Miller MF, Garfunkel AA, Ram C, Ship II. Inheritance patterns in recurrent aphthous ulcers: twin and pedigree data. *Oral Surg Oral Med Oral Pathol* 1977; **43**: 886–891.
11. Miller MF, Garfunkel AA, Ram CA, Ship II. The inheritance of recurrent aphthous stomatitis. Observations on susceptibility. *Oral Surg Oral Med Oral Pathol* 1980; **49**: 409–412.
12. Ship II. Epidemiologic aspects of recurrent aphthous ulcerations. *Oral Surg Oral Med Oral Pathol* 1972; **33**: 400–406.
13. Ślebioda Z, Szponar E, Kowalska A. Recurrent aphthous stomatitis: genetic aspects of etiology. *Postepy Dermatol Alergol* 2013; **30**: 96–102.
14. Borra RC, Andrade PM, Silva ID, Morgun A, Weckx LL, Smirnova AS, Franco M. The Th1/Th2 immune-type response of the recurrent aphthous ulceration analyzed by cDNA microarray. *J Oral Pathol Med* 2004; **33**: 140–146.
15. Han J, He Z, Li K, Hou L. Microarray analysis of potential genes in the pathogenesis of recurrent oral ulcer. *Int J Clin Exp Pathol* 2015; **8**: 12419–12427.
16. Bazrafshani MR, Hajeer AH, Ollier WE, Thornhill MH. IL-1B and IL-6 gene polymorphisms encode significant risk for the development of recurrent aphthous stomatitis (RAS). *Genes Immun* 2002; **3**: 302–305.
17. Bazrafshani MR, Hajeer AH, Ollier WE, Thornhill MH. Polymorphisms in the IL-10 and IL-12 gene cluster and risk of developing recurrent aphthous stomatitis. *Oral Dis* 2003; **9**: 287–291.

18. Ślebioda Z, Kowalska A, Rozmiarek M, Krawiecka E, Szponar E, Dorocka-Bobkowska B. The absence of an association between Interleukin 1β gene polymorphisms and recurrent aphthous stomatitis (RAS). *Arch Oral Biol* 2017: **84**: 45–49.
19. Izakovicova Holla L, Valova S, Borilova Linhartova P, Bartova J, Petanova J, Kuklinek P, Fassmann A. Association study of interleukin-1 family, interleukin-6, and its receptor gene polymorphisms in patients with recurrent aphthous stomatitis. *J Oral Pathol Med* 2017; **46**: 1030–1035.
20. Reichart PA. Oral mucosal lesions in a representative cross-sectional study of aging Germans. *Community Dent Oral Epidemiol* 2000; **28**: 390–398.
21. Pentenero M, Broccoletti R, Carbone M, Conrotto D, Gandolfo S. The prevalence of oral mucosal lesions in adults from the Turin area. *Oral Dis* 2008; **14**: 356–366.
22. Mansour Ghanaei F, Joukar F, Rabiei M, Dadashzadeh A, Kord Valeshabad A. Prevalence of oral mucosal lesions in an adult Iranian population. *Iran Red Crescent Med J* 2013; **15**: 600–604.
23. Feng J, Zhou Z, Shen X, Wang Y, Shi L, Wang Y, Hu Y, Sun H, Liu W. Prevalence and distribution of oral mucosal lesions: a cross-sectional study in Shanghai, China. *J Oral Pathol Med* 2015; **44**: 490–494.
24. Dalghous AM, Freysdottir J, Fortune F. Expression of cytokines, chemokines, and chemokine receptors in oral ulcers of patients with Behcet's disease (BD) and recurrent aphthous stomatitis is Th1-associated, although Th2-association is also observed in patients with BD. *Scand J Rheumatol* 2006; **35**: 472–475.
25. Ozyurt K, Celik A, Sayarlioglu M, Colgecen E, Inci R, Karakas T, Kelles M, Cetin GY. Serum Th1, Th2 and Th17 cytokine profiles and alpha-enolase levels in recurrent aphthous stomatitis. *J Oral Pathol Med* 2014; **43**: 691–695.
26. Sun A, Chu CT, Liu BY, Wang JT, Leu JS, Chiang CP. Expression of interleukin-2 receptor by activated peripheral blood lymphocytes upregulated by the plasma level of interleukin-2 in patients with recurrent aphthous ulcers. *Proc Natl Sci Counc Repub China B* 2000; **24**: 116–122.
27. Miyamoto NT Jr, Borra RC, Abreu M, Weckx LL, Franco M. Immune-expression of HSP27 and IL-10 in recurrent aphthous ulceration. *J Oral Pathol Med* 2008; **37**: 462–467.
28. Avci E, Akarslan ZZ, Erten H, Coskun-Cevher S. Oxidative stress and cellular immunity in patients with recurrent aphthous ulcers. *Braz J Med Biol Res* 2014; **47**: 355–360.
29. Ship JA, Chavez EM, Doerr PA, Henson BS, Sarmandi M. Recurrent aphthous stomatitis. *Quintessence Int* 2000; **31**: 95–112.
30. Karakus N, Yigit S, Rustemoglu A, Kalkan G, Bozkurt N. Effects of interleukin (IL)-6 gene polymorphisms on recurrent aphthous stomatitis. *Arch Dermatol Res* 2014; **306**: 173–180.
31. Slezakova S, Borilova Linhartova P, Masopustova L, Bartova J, Petanova J, Kuklinek P, Fassmann A, Dusek L, Izakovicova Holla L. Association of the NOD-like receptor 3 (NLRP3) gene variability with recurrent aphthous stomatitis in the Czech population. *J Oral Pathol Med* 2018; **47**: 434–439.
32. Mout R, Willemze R, Landegent JE. Repeat polymorphisms in the interleukin-4 gene (IL4). *Nucleic Acids Res* 1991; **19**: 3763.
33. Bartova J, Borilova Linhartova P, Podzimek S, Janatova T, Svobodova K, Fassmann A, Duskova J, Belacek J, Izakovicova Holla L. The effect of IL-4 gene polymorphisms on cytokine production in patients with chronic periodontitis and in healthy controls. *Mediators Inflamm* 2014; **2014**: 185757.
34. Padyukov L, Hahn-Zoric M, Lau YL, Hanson LA. Different allelic frequencies of several cytokine genes in Hong Kong Chinese and Swedish Caucasians. *Genes Immun* 2001; **2**: 280–283.
35. Lokossou AG, Dechavanne C, Bouraïma A, Courtin D, Le Port A, Ladékpo R, Noukpo J, Bonou D, Ahouangninou C, Sabbagh A, Fayomi B, Massougbodji A, Garcia A, Migot-Nabias F. Association of IL-4 and IL-10 maternal haplotypes with immune responses to P. falciparum in mothers and newborns. *BMC Infect Dis* 2013; **13**: 215.
36. Kalpana R, Thubashini M, Sundharam BS. Detection of salivary interleukin-2 in recurrent aphthous stomatitis. *J Oral Maxillofac Pathol* 2014; **18**: 361–364.
37. Pekiner FN, Aytugar E, Demirel GY, Borahan MO. Interleukin-2, interleukin-6 and T regulatory cells in peripheral blood of patients with Behçet's disease and recurrent aphthous ulcerations. *J Oral Pathol Med* 2012; **41**: 73–79.
38. Sun M, Fu SM, Dong GY, Wu D, Wang GX, Wu Y. Inflammatory factors gene polymorphism in recurrent oral ulceration. *J Oral Pathol Med* 2013; **42**: 528–534.
39. Najafi S, Yousefi H, Mohammadzadeh M, Bidoki AZ, Farhadi E, Rezaei N. Interleukin-2, interferon-gamma gene polymorphisms in recurrent aphthous stomatitis. *Prague Med Rep* 2017; **118**: 81–86.
40. May RD, Fung M. Strategies targeting the IL-4/IL-13 axes in disease. *Cytokine* 2015; **75**: 89–116.
41. Lewkowicz N, Kur B, Kurnatowska A, Tchorzewski H, Lewkowicz P. Expression of Th1/Th2/Th3/Th17-related genes in recurrent aphthous ulcers. *Arch Immunol Ther Exp (Warsz)* 2011; **59**: 399–406.
42. Wynn TA. IL-13 effector functions. *Annu Rev Immunol* 2003; **21**: 425–456.
43. Almoznino G, Zini A, Mizrahi Y, Aframian DJ. Elevated serum IgE in recurrent aphthous stomatitis and associations with disease characteristics. *Oral Dis* 2014; **20**: 386–394.
44. Cameron L, Webster RB, Strempel JM, Kiesler P, Kabesch M, Ramachandran H, Yu L, Stern DA, Graves PE, Lohman IC, Wright AL, Halonen M, Klimecki WT, Vercelli D. Th2 cell-selective enhancement of human IL13 transcription by IL13-1112C>T, a polymorphism associated with allergic inflammation. *J Immunol* 2006; **177**: 8633–8642.
45. Smolkova B, Tulinska J, Palkovicova Murinova L, Buocikova V, Liskova A, Rausova K, Kuricova M, Patayova H, Sustrova M, Neubauerova Svorcova E, Ilavska S, Szabova M, Nemessanyi T, Jahnova E, Dusinska M, Ciznar P, Fuortes L. Impact of IL13 genetic polymorphism Arg130Gln on total serum IgE levels and IFN-γ gene expression. *Clin Exp Immunol* 2017; **188**: 45–52.
46. Rosenwasser LJ, Klemm DJ, Dresback JK, Inamura H, Mascali JJ, Klinnert M, Borish L. Promoter polymorphisms in the chromosome 5 gene cluster in asthma and atopy. *Clin Exp Allerg* 1995; **25**: 74–78; discussion 95–96.
47. He JQ, Chan-Yeung M, Becker AB, Dimich-Ward H, Ferguson AC, Manfreda J, Watson WT, Sandford AJ. Genetic variants of the IL13 and IL4 genes and atopic diseases in at-risk children. *Genes Immun* 2003; **4**: 385–389.
48. Kalkan G, Yigit S, Karakus N, Baş Y, Seçkin HY. Association between interleukin 4 gene intron 3 VNTR polymorphism and recurrent aphthous stomatitis in a cohort of Turkish patients. *Gene* 2013; **527**: 207–210.
49. Buchs N, Silvestri T, Di Giovine FS, Chabaud M, Vannier E, Duff GW, Miossec P. IL-4 VNTR gene polymorphism in chronic polyarthritis. The rare allele is associated with protection against destruction. *Rheumatology (Oxford)* 2000; **39**: 1126–1131.
50. Oleksyk TK, Shrestha S, Truelove AL, Goedert JJ, Donfield SM, Phair J, Mehta S, O'Brien SJ, Smith MW. Extended IL10 haplotypes and their association with HIV progression to AIDS. *Genes Immun* 2009; **10**: 309–322.
51. Buño IJ, Huff JC, Weston WL, Cook DT, Brice SL. Elevated levels of interferon gamma, tumor necrosis factor alpha, interleukins 2, 4, and 5, but not interleukin 10, are present in recurrent aphthous stomatitis. *Arch Dermatol* 1998; **134**: 827–831.
52. Albanidou-Farmaki E, Markopoulos AK, Kalogerakou F, Antoniades DZ. Detection, enumeration and characterization of T helper cells secreting type 1 and type 2 cytokines in patients with recurrent aphthous stomatitis. *Tohoku J Exp Med* 2007; **212**: 101–105.

53. Jurge S, Kuffer R, Scully C, Porter SR. Mucosal disease series. Number VI. Recurrent aphthous stomatitis. *Oral Dis* 2006; **12**: 1–21.
54. Hu J, Hou S, Zhu X, Fang J, Zhou Y, Liu Y, Bai L, Kijlstra A, Yang P. Interleukin-10 gene polymorphisms are associated with Behcet's disease but not with Vogt-Koyanagi-Harada syndrome in the Chinese Han population. *Mol Vis* 2015; **21**: 589–603.
55. Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ, Hutchinson IV. An investigation of polymorphism in the interleukin-10 gene promoter. *Eur J Immunogenet* 1997; **24**: 1–8.
56. Koss K, Satsangi J, Fanning GC, Welsh KI, Jewell DP. Cytokine (TNF alpha, LT alpha and IL-10) polymorphisms in inflammatory bowel diseases and normal controls: differential effects on production and allele frequencies. *Genes Immun* 2000; **1**: 185–190.
57. Reuss E, Fimmers R, Kruger A, Becker C, Rittner C, Hohler T. Differential regulation of interleukin-10 production by genetic and environmental factors - a twin study. *Genes Immun* 2002; **3**: 407–413.
58. Mangia A, Santoro R, Piattelli M, Pazienza V, Grifa G, Iacobellis A, Andriulli A. IL-10 haplotypes as possible predictors of spontaneous clearance of HCV infection. *Cytokine* 2004; **25**: 103–109.
59. Najafi S, Firooze Moqadam I, Mohammadzadeh M, Bidoki AZ, Yousefi H, Farhadi E, Tonekaboni A, Meighani G, Amirzargar AA, Rezaei N. Interleukin-10 gene polymorphisms in recurrent aphthous stomatitis. *Immunol Invest* 2014; **43**: 405–409.
60. Guimarães AL, Correia-Silva Jde F, Sá AR, Victoria JM, Diniz MG, Costa Fde O, Gomez RS. Investigation of functional gene polymorphisms IL-1beta, IL-6, IL-10 and TNF-alpha in individuals with recurrent aphthous stomatitis. *Arch Oral Biol* 2007; **52**: 268–272.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Polymorphisms in genes encoding interleukins and their receptors. Results of case–control association studies with RAS patients.

Table S1

Polymorphisms in genes encoding interleukins and their receptors. Results of case-control association studies with RAS patients.

Gene	Polymorphism (rs number)	Reference	N (RAS patients/ controls)	Population/ (Race)	MAF (in the patient/ control group) *Frequency of genotype associated with RAS (in the patient control group)*	P value	OR (95%CI)	Association with RAS
IL-1	*IL-1A* -889 C/T (rs1800587)	(19)	64/184	Czech (Caucasian)	0.31/0.30 (T allele)	0.52	1.01 (0.66-1.57)	NS
		(61)	60/136	Iranian	0.34/0.32 (T allele)	0.703	1.12 (0.69–1.81)	NS
		(62)	39/48	Turkish	0.21/0.38 (T allele)	0.015	2.3	C allele is risk for RAS
		(16)	91/86	UK (Caucasian)	0.28/0.34 (T allele)	>0.05	–	NS
	IL-1A +4845 G/T (rs17561)	(16)	91/83	UK (Caucasian)	0.36/0.33 (T allele)	>0.05	–	NS
	IL-1B -511 T/C	(19)	64/184	Czech (Caucasian)	0.32/0.33 (T allele)	0.43	0.94 (0.61-1.44)	NS

1

(rs16944)	(18)	104/75	Polish (Caucasian)	0.28/0.37 (C allele) *0.52 0.36 (TT genotype)*	0.073 0.0347	– 1.92	TT genotype is risk for RAS
	(63)	36/130	Turkish	0.51/0.44 (T allele)	0.28	–	NS
	(61)	60/139	Iranian	0.48/0.45 (T allele)	0.564	1.16 (0.74–1.83)	NS
	(62)	41/56	Turkish	0.48/0.36 (T allele)	0.09	1.6 (for C allele)	NS
	(16)	91/91	UK (Caucasian)	0.51/0.29 (C allele) *0.28 0.08 (CC genotype)*	<0.00002 <0.0005	2.5 (1.6–4.0) 4.5 (1.9–10.9)	C allele and CC genotype are risk for RAS
IL-1B +3954 C/T (rs1143634)	(19)	64/184	Czech (Caucasian)	0.23/0.27 (T allele)	0.22	0.81 (0.50-1.30)	NS
	(18)	104/75	Polish (Caucasian)	0.23/0.23 (T allele)	0.9274	–	NS
	(64)	264/263	Chinese (Asian)	0.24/0.17 (T allele) *0.11 0.05 (TT genotype)*	– 0.002	– 2.86 (1.37-6.33)	TT genotype is risk for RAS
	(61)	60/140	Iranian	0.26/0.29 (T allele)	0.560	0.84 (0.50–1.40)	NS
	(62)	40/56	Turkish	0.14/0.30 (T allele)	0.015	2.6	C allele is risk for RAS

		(60)	64/64	Brazilian	0.28/0.18 (T allele)	–	–	CT genotype is risk for RAS
					0.56/0.36 (CT genotype)	0.03	2.40 (1.11- 5.20)	
		(16)	91/79	UK (Caucasian)	0.41/0.34 (T allele)	>0.05	–	NS
	IL-1R PstI +1970 C/T (rs2234650)	(61)	59/140	Iranian	0.36/0.44 (T allele)	0.831	0.93 (0.58–1.48)	NS
	IL-1RN 86 bp VNTR intron 2 (rs2234663)	(19)	64/184	Czech (Caucasian)	0.29/0.27 (allele 2 with 2 repeats)	0.39	1.09 (0.70-1.70)	NS
		(16)	91/91	UK (Caucasian)	0.26/0.35 (allele 2 with 2 repeats)	–	–	allele 1 with 4 repeats and 11 genotype are risk for RAS
					0.50/0.33 (11 genotype with 4 repeats)	<0.02	2.0 (1.1–3.6);	
	IL-1RN MspaI +11100 T/C (rs315952)	(61)	60/140	Iranian	0.20/0.23 (C allele)	0.616	0.84 (0.48–1.47)	NS
IL-2	*IL-2* -330 A/C (rs2069762)	(38)	42/86	Chinese (Asian)	0.32/0.26 (A allele)	0.3177	1.337 (0.756–2.365)	NS

		(39)*	60/140	Iranian	0.48/0.40 (C allele) *0.08 0.27 (AA genotype)* *0.87 0.68 (AC genotype)*	0.1290 0.0066 0.0089	1.43 (0.91–2.25) 0.25 (0.08–0.72) 3.11 (1.29–7.76)	AA genotype is protective for RAS and AC genotype is risk for RAS
	IL-2 +166 A/C (rs2069763)	(39)*	60/140	Iranian	0.33/0.21 (A allele) *0.37 0.59 (CC genotype)* *0.60 0.40 (AC genotype)*	0.0147 0.0061 0.0124	1.86 (1.12–3.07) 0.40 (0.21–0.79) 2.29 (1.18–4.46)	A allele and AC genotype is risk for RAS CC genotype is protective for RAS
IL-4	70 bp VNTR intron 3 (rs79071878)	(48)	145/150	Turkish	0.19/0.05 (allele 2 with 2 repeats) *0.66 0.90 (33 genotype with 3 repeats)*	<0.0001 –	0.25 (0.14-0.44) (for allele 3) –	allele 3 with 3 repeats and 33 genotype are protective for RAS
	-590 C/T (rs2243250)	(38)	42/86	Chinese (Asian)	0.46/0.45 (C allele)	0.8707 (for T allele)	0.957 (0.567–1.616)	NS
IL-6	-174 G/C (rs1800795)	(19)	64/184	Czech (Caucasian)	0.45/0.44 (C allele)	0.44	0.95 (0.63-1.42) (for G allele)	NS
		(64)	264/264	Chinese (Asian)	0.25/0.21 (C allele)	>0.05	–	NS

	(63)	36/130	Turkish	0.21/0.24 (C allele)	0.75	–	NS
	(61)	60/139	Iranian	0.41/0.36 (C allele) *0.10 0.03 (CC genotype)*	0.460 0.0441	1.21 (0.76–1.92) 3.75 (0.89–16.59)	CC genotype is risk for RAS
	(30)	184/150	Turkish	0.14/0.39 (C allele) *0.76 0.39 (GG genotype)*	<0.0001 <0.0001	3.82 (2.64–5.59) (for G allele) 4.87 (3.06–7.85)	G allele and GG genotype are risk for RAS
	(60)	64/64	Brazilian	0.21/0.19 (C allele)	–	–	NS
	(16)	91/91	UK (Caucasian)	0.20/0.40 (C allele) *0.64 0.34 (GG genotype)*	<0.0001 <0.0001	2.6 (1.6–4.1) (for G allele) 3.4 (1.9–6.2)	G allele and GG genotype are risk for RAS
-572 G/C (rs1800796)	(19)	64/184	Czech (Caucasian)	0.07/0.07 (C allele)	0.49	0.92 (0.42-2.04) (for G allele)	NS
	(30)**	184/150	Turkish	0.11/0.21 (C allele) *0.78 0.69 (GG genotype)*	0.0008 0.049	2.06 (1.35–3.17) (for G allele) 1.64 (1.00–2.69)	G allele and GG genotype are risk for RAS

		(16)	91/88	UK (Caucasian)	0.08/0.09 (C allele)	>0.05	–	NS
	-597 G/A (rs1800797)	(19)	64/184	Czech (Caucasian)	0.45/0.43 (A allele)	0.36	0.91 (0.61-1.36) (for G allele)	NS
		(61)	60/139	Iranian	0.18/0.18 (A allele)	0.978	0.97 (0.53–1.75)	NS
IL-6R	+48992 (rs2228145)	(19)	64/179	Czech (Caucasian)	0.36/0.37 (C allele)	0.42	0.94 (0.62-1.43)	NS
IL-10	-1082 A/G (rs1800896)	(64)	264/264	Chinese (Asian)	0.40/0.33 (G allele) *0.20 0.14 (GG genotype)*	– 0.03	– 1.72 (1.02-2.89)	GG genotype is risk for RAS
		(59)***	60/140	Iranian	0.39/0.35 (G allele) *0.75 0.54 (AG genotype)*	0.54 0.0074	1.18 (0.74–1.87) 2.60 (1.27–5.39)	AG genotype is risk for RAS
		(38)	42/86	Chinese (Asian)	0.23/0.29 (A allele)	0.3181	0.734 (0.399–1.349)	NS
		(60)	64/64	Brazilian	0.31/0.34 (G allele)	>0.05	–	NS
		(17)	100/91	UK (Caucasian)	0.46/0.47 (A allele)	0.8597.	0.96 (0.6–1.4)	NS

	-819 C/T (rs1800871)	(64)	264/264	Chinese (Asian)	0.44/0.41 (T allele)	>0.05	–	NS
		(59)***	57/140	Iranian	0.44/0.29 (T allele) *0.23 0.51 (CC genotype)* *0.67 0.41 (CT genotype)*	0.0062 (>0.05 after Bonferroni correction) 0.0005 0.0016	1.92 (1.19–3.09) 0.29 (0.13–0.61) 2.91 (1.46–5.85)	CC genotype is protective for RAS CT genotype is risk for RAS
	-592 C/A (rs1800872)	(59)***	58/140	Iranian	0.47/0.29 (A allele) *0.19 0.51 (CC genotype)* *0.67 0.41 (CA genotype)*	0.0006 <0.0001 0.0012	2.22 (1.38–3.55) 0.23 (0.10–0.50) 2.99 (1.50–6.00)	CC genotype is protective for RAS CA genotype is risk for RAS
		(38)	42/86	Chinese (Asian)	0.32/0.32 (A allele)	0.9787	1.008 (0.576–1.762)	NS
		(17)	100/91	UK (Caucasian)	0.20/0.20 (A allele)	0.4686	0.83 (0.5–1.4)	NS
IL-12	*IL-12A* T/G (rs2243115)	(38)	42/86	Chinese (Asian)	0.37/0.34 (T allele)	0.6155	1.150 (0.667–1.981)	NS

	IL-12A G/A (rs568408)	(38)	42/86	Chinese (Asian)	0.17/0.12 (A allele)	0.2647 (for G allele)	0.658 (0.314–1.378)	NS
	IL-12B +1188T/G (rs3212227)	(65)	59/140	Iranian	0.23/0.27 (G allele)	0.446	0.80(0.47-1.36)	NS
		(38)	42/86	Chinese (Asian)	0.20/0.30 (T allele)	0.0906	0.586 (0.314–1.093)	NS
		(17)	100/91	UK (Caucasian)	0.23/0.18 (G allele)	0.2407	0.74 (0.5–1.2)	NS
IL-18	-607 G/T (rs1946518)	(66)	80/80	Egyptian	0.46/0.49 (T allele)	0.65	0.9 (0.58–1.4)	NS
	-137 G/C (rs187238)	(66)	80/80	Egyptian	0.32/0.37 (C allele)	0.35	0.8 (0.5–1.3)	NS

IL=interleukin, RAS=recurrent aphthous stomatitis, R=receptor, RN=receptor antagonist, NS=non-significant difference, VNTR=variable number of tandem repeat, –=unknown

*AC haplotype is risk for RAS (P=0.0345; OR= 0.64; 95% CI=0.42–0.97)

**GG haplotype ($P=1.5 \times 10^{-8}$; OR=3.52; 95% CI=2.54–4.87) and GG/GG haplogenotype (P<0.0001) are risk for RAS

***ACC haplotype is protective for RAS (P<0.0001; OR=0.2; 95% CI= 0.1-0.43)

References

61. NAJAFI S, YOUSEFI H, MOHAMMADZADEH M, BIDOKI AZ, FIROUZE MOQADAM I, FARHADI E, AMIRZARGAR AA, REZAEI N. Association study of interleukin-1 family and interleukin-6 gene single nucleotide polymorphisms in recurrent aphthous stomatitis. *Int J Immunogenet* 2015; **42**: 428–431.

62. AKMAN A, EKINCI NC, KACAROGLU H, YAVUZER U, ALPSOY E, YEGIN O. Relationship between periodontal findings and specific polymorphisms of interleukin-1alpha and -1beta in Turkish patients with Behçet's disease. *Arch Dermatol Res* 2008; **300**: 19–26.

63. YAKAR T, SERIN E, COŞAR AM, ARSLAN TAŞ D, ATAÇ FB. The relationship of recurrent aphthous stomatitis and Helicobacter pylori, cytokine gene polymorphism and cobalamin. *Turk J Gastroenterol* 2015; **26**: 304–308.

64. JING C, ZHANG JQ. Association between interleukin gene polymorphisms and risk of recurrent oral ulceration. *Genet Mol Res 2015*; **14**: 6838–6843.

65. FIROUZE MOQADAM I, NAJAFI S, MOHAMMADZADEH M, ZARE BIDOKI A, YOUSEFI H, FARHADI E, TONEKABONI A, MEIGHANI G, MOHAMMADZADEH M, AMIRZARGAR AA, REZAEI N. Lack of association between interleukin-12 gene polymorphisms and recurrent aphthous stomatitis. *Avicenna J Med Biotechnol* 2016; **8**: 200–201.

66. HAZZAA HHA, RASHWAN WAM, ATTI EAS. IL-18 gene polymorphisms in aphthous stomatitis vs. Behcet's disease in a cohort of Egyptian patients. *J Oral Pathol Med* 2014; **43**: 746–753.

1.2 Comment to the article "Association study of interleukin-1 family, interleukin-6, and its receptor gene polymorphisms in patients with recurrent aphthous stomatitis"

Izakovicova Holla L, Valova S, Borilova Linhartova P, Bartova J, Petanova J, Kuklinek P, Fassmann A. Association study of interleukin-1 family, interleukin-6, and its receptor gene polymorphisms in patients with recurrent aphthous stomatitis. J Oral Pathol Med. 2017;46(10):1030-1035. DOI: 10.1111/jop.12594. (IF 2.237; DENTISTRY, ORAL SURGERY & MEDICINE Q1, PATHOLOGY Q2)

Author contribution: P.B.L. particularly carried out the genetic analysis and revised the manuscript.

IL-1 is a proinflammatory cytokine responsible for the induction of other cytokines and chemokines. The IL-1 cytokine family consists of three structurally related proteins, of which two are agonists (IL-1 alpha and IL-1 beta) and the third, IL-1 receptor antagonist (IL-1RA), is a competitive antagonist. IL-1RA binds to IL-1R with a higher affinity than IL-1 and leads to regulation of cytokine secretion.

IL-6 is a multifunctional cytokine that participates in inflammatory response; it is especially important for the acute phase response. IL-6 acts through a receptor comprising two subunits, IL-6 receptor (IL-6R) and glycoprotein 130.

Several studies have evaluated the potential association between *IL-1A, IL-1B*, *IL-1RN*, or *IL-6* gene polymorphisms and an increased susceptibility to aphthous stomatitis with conflicting results. To date, no study has been conducted on the *IL-6R* gene variants in this disease (see Overview in the Table S1 on the page 28).

In this study, we aimed to examine the associations between the *IL-1* family, *IL-6*, and its receptor gene variants alone or in combinations (so-called *IL-1* and *IL-6* haplotypes) and RAS in the Czech population.

A total of 248 subjects, 64 patients with RAS and 184 healthy controls were genotyped for *IL-1A* rs1800587, *IL-1B* rs16944, and *IL-1B* rs1143634, *IL-6* rs1800797, *IL-6* rs1800796, *IL-6* rs1800795, and *IL-6R* rs2228145 SNPs, and *IL-1RN* variable tandem repeats (VNTR) in intron 2.

No significant differences between the investigated polymorphisms in patients with RAS and the healthy subjects were detected (P>0.05). Patients with AC genotype *IL-6R* rs2228145 had a marginally, but non-significantly decreased risk of developing RAS (P=0.09, OR=0.61, 95%CI=0.32-1.15) in comparison with AA homozygotes. In addition,

a complex analysis also revealed similar *IL-1* or *IL-6* haplotype frequencies between both groups ($P>0.05$).

Thus *IL-1* gene cluster and *IL-6* or its receptor gene variants cannot be used as markers for the identification of Czech patients with an increased risk of RAS. Very conflicting results regarding the association of these *IL-1* and *IL-6* gene variants with RAS have been detected across different populations.

Accepted: 16 May 2017
DOI: 10.1111/jop.12594

ORIGINAL ARTICLE

WILEY Journal of Oral Pathology & Medicine

Association study of *interleukin-1* family, *interleukin-6*, and *its receptor* gene polymorphisms in patients with recurrent aphthous stomatitis

Lydie Izakovicova Holla[1,2] | Simona Valova[1,2] | Petra Borilova Linhartova[1,2] | Jirina Bartova[3] | Jitka Petanova[4] | Pavel Kuklinek[5] | Antonin Fassmann[1]

[1]Clinic of Stomatology, Institution Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Brno, Czech Republic
[2]Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic
[3]Institute of Clinical and Experimental Dental Medicine, General University Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic
[4]Department of Immunology and Microbiology, General University Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic
[5]Department of Clinical Immunology and Allergology, Institution Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Brno, Czech Republic

Correspondence
Lydie Izakovicova Holla, Clinic of Stomatology and Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.
Email: holla@med.muni.cz

Funding information
Ministerstvo Zdravotnictví Ceské Republiky, Grant/Award Number: AZV15-29336A

Background: Recurrent aphthous stomatitis (RAS) is one of the most common oral chronic ulcerative disease in which proinflammatory cytokines such as interleukin-1 (IL-1) and interleukin-6 (IL-6) are thought to play an important role. The aim of this study was to investigate the possible association between polymorphisms in the IL-1 cytokine family, IL-6 or its receptor and RAS in the Czech population.

Methods: A total of 248 subjects, 184 healthy controls, and 64 patients with RAS were genotyped for *IL-1A*-889C>T, *IL-1B*-511C>T, *IL-1B*+3953C>T, *IL-1RN*86 bp variable number of tandem repeats (VNTRs) in intron 2, *IL-6*-597G>A, *IL-6*-572G>C, *IL-6*-174G>C, and *IL-6R*+48992A>C by polymerase chain reaction (PCR) methods.

Results: No significant differences between investigated polymorphisms in healthy subjects and patients with RAS were detected (P>.05). In addition, complex analysis also revealed similar *IL-1* or *IL-6* haplotype frequencies between both groups (P>.05).

Conclusions: In conclusion, *IL-1* and *IL-6* or *its receptor* gene variants cannot be used as markers for identification of Czech patients with increased risk of recurrent aphthous stomatitis.

KEYWORDS
aphthous stomatitis, case-control study, gene polymorphism, interleukin-1, interleukin-6

1 | INTRODUCTION

Recurrent aphthous stomatitis (RAS, recurrent aphthous ulcers, canker sores) is a common multifactorial disease characterized by recurrent episodes of oral ulcerations. The prevalence of RAS is between 5% and 20% in the general population.[1] The disease manifests as round or ovoid, painful, self-healing, and recurrent ulcers with circumscribed margins and erythematous haloes[2] and can be classified into three clinical forms: minor, major, and herpetic ulcers. The etiology of RAS is unknown; it has been suggested that immune dysfunction, stress, local trauma, nutritional factors, hormonal changes, and also genetic disposition can be important.[3,4] The role of genetic factors in etiopathogenesis of RAS has been confirmed in studies of relatives and twins, where the positive family history of the disease was reported in 24%-46% of cases.[5] In predisposed patients, the effect of certain trigger factors initiates the cascade of proinflammatory cytokines, such as interleukin-1 (IL-1) or interleukin-6 (IL-6), and leads to immune dysregulation.[6,7]

IL-1 is a proinflammatory cytokine responsible for the induction of other cytokines and chemokines. The IL-1 cytokine family consists of three structurally related proteins, of which two are agonists (IL-1α and IL-1β) and the third, IL-1 receptor antagonist (IL-1RA), is a

© 2017 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

competitive antagonist. IL-1RA binds to IL-1R with higher affinity than IL-1 and leads to ablation of cytokine secretion.[8] These proteins are encoded by a gene cluster located on a chromosome 2q14 containing several polymorphic regions. Polymorphism at position −889C>T of *IL-1A* has been described to have a regulatory effect on basal IL-1β production[9] and possible overexpression of IL-1α.[10] *IL-1B* promoter single nucleotide polymorphisms (SNPs, including -511C>T) have been indicated to regulate the transcriptional activity of the *IL-1B* gene.[11] The *IL-1RN* gene exists in six allelic variants corresponding to one to six copies of the 86-bp sequence repeat. The 4-repeat (*IL-1RN**1) and 2-repeat (*IL-1RN**2) alleles are most common, whereas others occur at a combined frequency of <5%. The *IL-1RN* variability results in quantitative differences in both IL-1RA and IL-1β production.[12]

IL-6 is a multifunctional cytokine that participates in inflammatory response; it is especially important for the acute phase response. The *IL-6* gene is located on chromosome 7p21, and a number of polymorphisms have been detected in the promoter region of this gene. The *IL-6*-174G>C variant is located immediately upstream of a multiresponse element at position −173 to −151 and affects *IL-6* transcription.[13] Furthermore, two other functional SNPs, at positions −597G>A and -572G>C, have been reported to have an effect on IL-6 secretion capacity.[14] IL-6 acts through a receptor comprising two subunits, IL-6 receptor (IL-6R) and glycoprotein 130 (gp130). The *IL-6R* gene maps to human chromosome 1q21. The +48892A>C (Asp358Ala) polymorphism in exon 9 corresponds to the proteolytic cleavage site of IL-6Rα. Subjects carrying CC genotype (Ala358Ala) had significantly higher serum levels of soluble IL-6R (sIL-6R).[15,16]

Several studies evaluated the potential association between *IL-1A*, *IL-1B*, *IL-1RN*, or *IL-6* gene polymorphisms and an increased susceptibility to aphthous stomatitis with conflicting results.[8,17-23] To date, no study has been conducted on the *IL-6R* gene variants in this disease.

Therefore, in this study, we aimed to examine the associations between the *IL-1* family, *IL-6*, and *its receptor* gene variants alone or in combinations (so-called *IL-1* and *IL-6* haplotypes) and RAS in the Czech population.

2 | MATERIALS AND METHODS

2.1 | Subjects

The study group consisted of 64 unrelated patients with RAS (36 males and 28 females; mean age: 40.2±15.0 standard deviation [SD] years) and 184 (92 males and 92 females; mean age: 46.5±11.8 SD years) unrelated healthy controls. All participants, patients and healthy controls, were of Czech origin. RAS patients were gathered from the Department of Immunology and Microbiology, General University Hospital and First Faculty of Medicine, Charles University, Prague, and Department of Clinical Immunology and Allergology, Institution Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Brno. The diagnosis of RAS was made by oral medicine specialists based on the accepted clinical criteria.[24] The control group consisted of age- and sex-matched healthy individuals without history of RAS. Exclusion criteria for both groups included the presence of any other significant local or systemic diseases: Behçet's disease, celiac disease and other gastrointestinal symptoms and/or diseases or pregnancy. The study protocol was approved by the Local Ethics Committee of Masaryk University, Faculty of Medicine (39/2015), St. Anne's Faculty Hospital (8G/2015), and General University Hospital and First Faculty of Medicine, Charles University, Prague (53/14), and written informed consent was obtained from the study participants in line with the Helsinki declaration prior to their inclusion in the study.

2.2 | Genotyping

Genomic DNA was purified from peripheral blood leukocytes by the standard method using the phenol-chloroform extraction and the proteinase K digestion of cells. Isolation, storage of DNA, and genotyping of samples were conducted in the laboratory of the Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.

Eight polymorphisms in the genes for *IL-1* and *its receptor antagonist* (*IL-1RN*), *IL-6*, and *its receptor* (*IL-6R*) were based on the polymerase chain reaction (PCR). SNPs *IL-1A* (-889C>T rs1800587), *IL-1B* (-511C>T rs16944), and *IL-1B* (+3953C>T rs1143634) were genotyped by PCR-RFLP (restriction fragment length polymorphism) according to the methods described previously.[25-27] The variable number of tandem repeat (VNTR) PCR technique was used for the detection of 86-bp repetitions in the intron 2 of the *IL-1RN* gene.[28] These methods were optimized, and details were presented in our previous study.[29]

Single nucleotide analysis of the *IL-6* gene (-597G>A rs1800797, -572G>C rs1800796, -174G>C rs1800795) was also based on the PCR-RFLP approach.[13,30] Amplification of DNA fragment containing polymorphism *IL-6R* (+48892A>C rs2228145) was carried out in reaction volume of 25.0 μL containing 75 ng of genomic DNA, 0.24 μmol/L of each primer,[15] 1.5 U of DNA polymerase (Thermo Fischer Scientific, Waltham, MA, USA), 3.5 mM of $MgCl_2$, 10× $MgCl_2$-free reaction buffer with $(NH_4)_2SO_4$, and 0.2 mM deoxyribonucleoside triphosphate mix (Thermo Fischer Scientific, Waltham, MA, USA). Denaturation for 5 minutes at 95°C was followed by 25 cycles of 95°C for 1 minute, 57°C for 1 minite, and 72°C for 1 minute in the thermocycler (SensoQuest, Schoeller Instruments). The last synthesis step was extended to 10 minutes at 72°C. The PCR products were then digested with *HinfI* restriction enzyme. The restriction was performed in a volume of 30.0 μL consisting of 25.0 μL of the PCR product, 10× CutSmart Buffer (New England Biolabs, Ipswich, MA, USA), and 5 U of *HinfI* enzyme (New England Biolabs, Ipswich, MA, USA), that is, 25 μL PCR product+0.1 μL enzyme+3 μL buffer+1.9 μL H_2O, and incubated overnight at 37°C. The digested products were electrophoresed in 2% agarose gel and visualized by ethidium bromide staining.

2.3 | Statistical analysis

Standard descriptive statistics were applied in the analysis: mean and standard deviation (SD) for quantitative variables and absolute and relative frequencies for categorical variables. The chi-square and Fisher's exact tests were used for comparison of differences in genotypes or allele frequencies among groups. Bonferroni correction was applied to adjust the level according to the number of independent comparisons to an overall value of P_{corr}<.05. Linkage disequilibrium was measured by Lewontin standardized disequilibrium coefficient (*D'*), and haplotypes were determined using the program SNP Analyzer 2 (available on http://snp.istech21.com/snpanalyzer/2.0/). Odds ratio (OR), confidence intervals (CIs), and *P* values were calculated. All statistical analyses were performed using the program package Statistica v. 12 (StatSoft Inc., Tulsa, OK, USA).

3 | RESULTS

The distributions of genotype and allele frequencies of *IL-1* family, *IL-6*, and *its receptor* in patients with RAS and healthy controls are shown in Table 1. Genotype frequencies of the studied polymorphisms were in Hardy-Weinberg equilibrium (HWE, *P*>.05); only the SNPs *IL-1*-511 in controls and *IL-6R*+48992 in patients with recurrent aphthae were not in the genetic balance in our population (HWE=0.99, *P*<.01).

None of these polymorphisms was found to be significantly associated with RAS (*P*>.05). Patients with *IL-6R*+48992 AC genotype had marginally but non-significantly decreased risk of developing RAS (*P*=.09, OR=0.61, 95% CI=0.32-1.15) in comparison with AA homozygotes.

Combination of multiple variable sites showed eleven *IL-1* haplotypes with frequency more than 1% because all four polymorphisms in the *IL-1* gene exhibited variability in the degree of linkage disequilibrium. The frequencies of these *IL-1* haplotypes are summarized in Table 2; no significant differences (*P*>.05) among *IL-1* haplotypes in Czech patients with RAS and healthy controls were found.

When we analyzed combination of three *IL-6* SNPs, only five haplotypes were found in our subjects. However, the distributions of *IL-6* haplotypes were also similar between both groups (Table 3).

4 | DISCUSSION

Recurrent aphthous stomatitis is a very common oral mucosal ulcer disease; however, its etiopathogenesis is not well understood. In this study, we evaluated several polymorphisms of the *IL-1* family, *IL-6*, and *its receptor* genes in a group of 248 Czech subjects. All the examined variants are located within the regulatory regions or in the coding sequence of the respective genes. They are of potential functional importance as they have been shown to modulate cytokine production.[9-12,14-16] Our data revealed no significant differences in frequencies of alleles or genotypes of the investigated variants among healthy and RAS-affected subjects. These findings are in disagreement with some previous studies that claimed that polymorphisms of selected cytokines were associated with an increased risk of RAS.[8,17-20,22] Jing and Zhang[22] described that *IL-1B*+3954 (according to different counting +3953 as used in this study) TT genotype was significantly associated with RAS. However, Guimarães et al.[18] showed that *IL-1B*+3954 CT heterozygotes can influence RAS development, and Akman and colleagues[19] found C allele of this polymorphism increased in patients with aphthous stomatitis. The same authors also reported significant association of the *IL-1A*-889 C allele with RAS. In addition, Bazrafshani et al.[17] found C allele and CC genotype of the *IL-1*-511C>T variant and allele 1 (with four repeats) and 11 genotypes of the *IL-1RN* 86-bp VNTR in intron 2 as risk factors for recurrent aphthae. Thus, obviously very conflicting results regarding the association of these *IL-1* gene variants with RAS have been detected. Similarly as in our study, no significant differences of the allele or genotype frequencies between healthy subjects and patients with RAS have been found in different studies for *IL-1A*-889C>T,[8,17] *IL-1B*-511C>T,[8,19,21,23] and *IL-1B*+3953C>T[8,17,23] polymorphisms.

In addition, a few studies on the relationship between *IL-6* promoter polymorphisms and RAS have been published; however, their results are also conflicting. While Bazrafshani et al.[17] and Karakus with colleagues[20] reported a positive association between carriage of the *IL-6*-174 GG genotype and G allele and RAS, recent study by Najafi et al.[8] found significantly higher frequency of *IL-6*-174 CC genotype in a group of patients with RAS. In contrast, other authors could not confirm any association between *IL-6* promoter variants and RAS in different populations.[18,21,22] It is in agreement with our findings where similar frequencies of the *IL-6* genotypes and/or alleles between patients with RAS and healthy controls were found. Differences among studies can be considered in relation to methodological approaches applied, sample size, and especially ethnic background. In the present study, we have examined for the first time the contribution of the *IL-6R*+48992A>C polymorphism in RAS. Although heterozygotes of this polymorphism had slightly decreased risk of developing RAS in comparison with common AA homozygotes, we were unable to find any significant association of this variant with RAS.

However, individual SNPs do not exist in isolation, but are genetically linked, forming distinct haplotypes. As haplotype analyses may be of a higher informative value for drawing associations between phenotypes and genetic variation than single polymorphisms, we inferred haplotypes in *IL-1* and *IL-6* from genotype data and performed haplotype-based association analysis with RAS. As far as we know, this is the first study that performed the haplotype analysis of four *IL-1A*, *IL-1B*, and *IL-1RN* gene variants in RAS. Haplotype analysis of *IL-6* gene variants was previously performed by Karakus et al.;[20] in other populations, only single variants were investigated. Karakus and colleagues[20] described that the *IL-6*-174 and -572 GG haplotype were found significantly higher in RAS patients and haplotype represented by the G allele for the -597 and -174 loci appeared to favor a progressive disease in Turkey population. In contrast, our

TABLE 1 The distribution of genotype and allele frequencies in healthy controls and patients with RAS

Gene	Genotypes/alleles	Healthy controls N (%)	Patients with RAS N (%)	*P* value	OR (95% CI)
IL-1A-889	CC	86 (46.7)	29 (45.3)	.94	1.00
	CT	85 (46.2)	31 (48.4)		1.08 (0.60-1.95)
	TT	13 (7.1)	4 (6.3)		0.91 (0.28-3.02)
	C	257 (69.8)	89 (69.5)	.52	1.00
	T	111 (30.2)	39 (30.5)		1.01 (0.66-1.57)
IL-1B-511	CC	89 (48.4)	31 (48.4)	.85	1.00
	CT	67 (36.4)	25 (39.1)		1.07 (0.58-1.98)
	TT	28 (15.2)	8 (12.5)		0.82 (0.34-1.99)
	C	245 (66.6)	87 (68.0)	.43	1.00
	T	123 (33.4)	41 (32.0)		0.94 (0.61-1.44)
IL-1B+3953	CC	97 (52.7)	37 (57.8)	.60	1.00
	CT	76 (41.3)	25 (39.1)		0.86 (0.48-1.56)
	TT	11 (6.0)	2 (3.1)		0.48 (0.10-2.25)
	C	270 (73.4)	99 (77.3)	.22	1.00
	T	98 (26.6)	29 (22.7)		0.81 (0.50-1.30)
IL-1RN VNTR[a]	11	90 (48.9)	29 (45.3)	.21	1.00
	12	70 (38.0)	27 (42.2)		1.20 (0.65-2.20)
	13	7 (3.8)	0 (0.0)		[b]
	14	2 (1.1)	3 (4.7)		4.66 (0.74-29.24)
	22	15 (8.2)	5 (7.8)		0.97 (0.32-2.89)
	L	268 (72.8)	91 (71.1)	.39	1.00
	S	100 (27.2)	37 (28.9)		1.09 (0.70-1.70)
IL-6-597	AA	35 (19.0)	11 (17.2)	.49	1.00
	AG	88 (47.8)	36 (56.3)		1.30 (0.60-2.84)
	GG	61 (33.2)	17 (26.6)		0.89 (0.37-2.11)
	A	158 (42.9)	58 (45.3)	.36	1.00
	G	210 (57.1)	70 (54.7)		0.91 (0.61-1.36)
IL-6-572	CC	0 (0.0)	0 (0.0)	.84	[b]
	CG	24 (13.0)	9 (14.1)		1.09 (0.48-2.49)
	GG	160 (87.0)	55 (85.9)		1.00
	C	24 (6.5)	9 (7.0)	.49	0.92 (0.42-2.04)
	G	344 (93.5)	119 (93.0)		1.00
IL-6-174	CC	35 (19.0)	11 (17.2)	.69	1.00
	CG	92 (50.0)	36 (56.3)		1.25 (0.57-2.71)
	GG	57 (31.0)	17 (26.6)		0.95 (0.40-2.26)
	C	162 (44.0)	58 (45.3)	.44	1.00
	G	206 (56.0)	70 (54.7)		0.95 (0.63-1.42)
IL-6R+48992[c]	AA	70 (39.1)	30 (46.9)	.21	1.00
	AC	84 (46.9)	22 (34.4)		0.61 (0.32-1.15)
	CC	25 (14.0)	12 (18.8)		1.12 (0.50-2.52)
	A	224 (62.6)	82 (64.1)	.42	1.00
	C	134 (37.4)	46 (35.9)		0.94 (0.62-1.43)

CI, confidential interval; IL, interleukin, IL-RN, interleukin receptor antagonist, OR, odds ratio, RAS, recurrent aphthous stomatitis, VNTRs, variable number of tandem repeats.

[a]For allele analysis, alleles with more than two repeat units were grouped together as *IL-1RN**L (L=long allele), 2-repeat allele was marked as *IL-1RN**L (S=short allele).

[b]cannot be assessed due to small number.

[c]Genotypes are known only in 179 controls.

TABLE 2 Estimated frequencies of *IL-1* haplotypes

Haplotypes				Healthy controls (N=184)	Patients with RAS (N=64)	P value	OR (95% CI)
IL-1A-889C>T	*IL-1B-511C>T*	*IL-1B+3953C>T*	*IL-1RNVNTR*[a]				
C	C	C	L	0.3372	0.3667	.4378	1.181 (0.777-1.793)
T	C	T	L	0.1970	0.2096	.9232	1.024 (0.629-1.668)
C	T	C	S	0.1626	0.1771	.8826	1.040 (0.615-1.760)
C	T	C	L	0.0974	0.0861	.5587	0.806 (0.387-1.679)
C	C	C	S	0.0693	0.0666	1.000	1.000 (0.436-2.295)
T	T	C	L	0.0261	0.0317	.9386	1.047 (0.327-3.348)
T	T	T	L	0.0260	0.0000	[b]	[b]
T	C	C	L	0.0223	0.0165	.5899	0.572 (0.066-4.940)
C	C	T	L	0.0207	0.0000	[b]	[b]
T	C	T	S	0.0107	0.0157	.7713	1.441 (0.130-16.027)
T	T	C	S	0.0106	0.0243	.1403	2.935 (0.723-11.914)
Others (<0.01%)				0.0202	0.0058	[b]	[b]

CI, confidential interval; IL, interleuki; IL-RN, interleukin receptor antagonist; OR, odds ratio; RAS, recurrent aphthous stomatitis; VNTRs, variable number of tandem repeats.
Haplotypes are ordered according to decreasing haplotype frequency in the healthy subjects.
[a]For haplotype analysis, alleles with more than two repeat units were grouped together as *IL-1RN**L (L=long allele), 2-repeat allele was marked as *IL-1RN**S (S=short allele).
[b]Cannot be assessed due to small number.

TABLE 3 Estimated frequencies of *IL-6* haplotypes

Haplotypes			Healthy controls (N=184)	Patients with RAS (N=64)	P value	OR (95% CI)
IL-6-597G>A	*IL-6-572G>C*	*IL-6-174G>C*				
G	G	G	0.4994	0.4766	.6862	0.920 (0.615-1.377)
A	G	C	0.4245	0.4531	.6027	1.114 (0.743-1.669)
G	C	G	0.0604	0.0703	.7587	1.134 (0.511-2.520)
G	G	C	0.0109	0.0000	[a]	[a]
A	C	C	0.0049	0.0000	[a]	[a]

Haplotypes are ordered according to decreasing haplotype frequency in the healthy subjects.
CI, confidential interval; IL, interleukin; OR, odds ratio; RAS, recurrent aphthous stomatitis.
[a]Cannot be assessed due to small number.

results suggest that *IL-6* haplotypes have no effect on the risk of RAS in the Czech population. We also found similar frequencies of *IL-1* haplotypes between groups of healthy subjects and patients with RAS.

There are some limitations of this study that need to be mentioned. The case-control approach used is vulnerable to the population stratification, for example, ethnic origin but our subjects were exclusively of the Czech Caucasian. Next, a relatively small number of patients with RAS limit the statistical power to find the differences between groups. However, our sample size is comparable with almost all previously published studies, except two larger studies by Jing & Zhang[22] and Karakus et al.[20] Finally, we studied only selected variants in the cytokine genes in RAS, a multifactorial disease in which interactions among multiple genes and environmental factors play a role.

In summary, although the results of some previous studies indicated that *IL-1* and/or *IL-6* gene variants might be associated with RAS, findings of our study suggest that the studied polymorphisms are not key factors for the development of this disease in the Czech population. Nevertheless, further investigations in larger populations with different ethnic background are needed to confirm this finding.

ACKNOWLEDGEMENTS

The study was supported by grant AZV15-29336A.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTION

L.I.H, J.P., and J.B. designed the study and drafted the manuscript. J.P., A.F., and P.K. performed the clinical analyses and collected

blood samples. P.B.L. and S.V. carried out the molecular analysis; L.I.H. performed statistical analysis. All authors revised the final version of the manuscript.

REFERENCES

1. Scully C, Porter S. Oral mucosal disease: recurrent aphthous stomatitis. *Br J Oral Maxillofac Surg*. 2008;46:198-206.
2. Jurge S, Kuffer R, Scully C, Porter SR. Mucosal disease series. Number VI. Recurrent aphthous stomatitis. *Oral Dis*. 2006;12:1-21.
3. Chavan M, Jain H, Diwan N, Khedkar S, Shete A, Durkar S. Recurrent aphthous stomatitis: a review. *J Oral Pathol Med*. 2012;41:577-583.
4. Ślebioda Z, Szponar E, Kowalska A. Recurrent aphthous stomatitis: genetic aspects of etiology. *Postepy Dermatol Alergol*. 2013;30:96-102.
5. Natah SS, Konttinen YT, Enattah NS, Ashammakhi N, Sharkey KA, Häyrinen-Immonen R. Recurrent aphthous ulcers today: a review of the growing knowledge. *Int J Oral Maxillofac Surg*. 2004;33:221-234.
6. Buño IJ, Huff JC, Weston WL, Cook DT, Brice SL. Elevated levels of interferon gamma, tumor necrosis factor alpha, interleukins 2, 4, and 5, but not interleukin 10, are present in recurrent aphthous stomatitis. *Arch Dermatol*. 1998;134:827-831.
7. Dinarello CA, Simon A, Meer JWM. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat Rev Drug Discov*. 2012;11:633-652.
8. Najafi S, Yousefi H, Mohammadzadeh M, et al. Association study of interleukin-1 family and interleukin-6 gene single nucleotide polymorphisms in recurrent aphthous stomatitis. *Int J Immunogenet*. 2015;42:428-431.
9. Hulkkonen J, Laippala P, Hurme M. A rare allele combination of the interleukin-1 gene complex is associated with high interleukin-1 beta plasma levels in healthy individuals. *Eur Cytokine Netw*. 2000;11:251-255.
10. Dominici R, Cattaneo M, Malferrari G, et al. Cloning and functional analysis of the allelic polymorphism in the transcription regulatory region of interleukin-1alpha. *Immunogenetics*. 2002;54:82-86.
11. Chen H, Wilkins LM, Nazneen A, et al. Single nucleotide polymorphisms in the human interleukin-1B gene affect transcription according to haplotype context. *Hum Mol Genet*. 2006;4:519-529.
12. Vamvakopoulos J, Green C, Metcalfe S. Genetic control of IL-1beta bioactivity through differential regulation of the IL-1 receptor antagonist. *Eur J Immunol*. 2002;32:2988-2996.
13. Fishman D, Faulds G, Jeffery R, et al. The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. *J Clin Invest*. 1998;102:1369-1376.
14. Terry CF, Loukaci V, Green FR. Cooperative influence of genetic polymorphisms on interleukin 6 transcriptional regulation. *J Biol Chem*. 2000;275:18138-18144.
15. Galicia JC, Tai H, Komatsu Y, Shimada Y, Akazawa K, Yoshie H. Polymorphisms in the IL-6 receptor (IL-6R) gene: strong evidence that serum levels of soluble IL-6R are genetically influenced. *Genes Immun*. 2004;5:513-516.
16. Rafiq S, Frayling TM, Murray A, et al. A common variant of the interleukin 6 receptor (IL-6r) gene increases IL-6r and IL-6 levels, without other inflammatory effects. *Genes Immun*. 2007;8:552-559.
17. Bazrafshani MR, Hajeer AH, Ollier WE, Thornhill MH. IL-1B and IL-6 gene polymorphisms encode significant risk for the development of recurrent aphthous stomatitis (RAS). *Genes Immun*. 2002;3:302-305.
18. Guimarães AL, Correia-Silva JdeF, Sá AR, et al. Investigation of functional gene polymorphisms IL-1beta, IL-6, IL-10 and TNF-alpha in individuals with recurrent aphthous stomatitis. *Arch Oral Biol*. 2007;52:268-272.
19. Akman A, Ekinci NC, Kacaroglu H, Yavuzer U, Alpsoy E, Yegin O. Relationship between periodontal findings and specific polymorphisms of interleukin-1alpha and -1beta in Turkish patients with Behçet's disease. *Arch Dermatol Res*. 2008;300:19-26.
20. Karakus N, Yigit S, Rustemoglu A, Kalkan G, Bozkurt N. Effects of interleukin (IL)-6 gene polymorphisms on recurrent aphthous stomatitis. *Arch Dermatol Res*. 2014;306:173-180.
21. Yakar T, Serin E, Coşar AM, Arslan Taş D, Ataç FB. The relationship of recurrent aphthous stomatitis and Helicobacter pylori, cytokine gene polymorphism and cobalamin. *Turk J Gastroenterol*. 2015;26:304-308.
22. Jing C, Zhang JQ. Association between interleukin gene polymorphisms and risk of recurrent oral ulceration. *Genet Mol Res*. 2015;14:6838-6843.
23. Ślebioda Z, Krawiecka E, Rozmiarek M, Szponar E, Kowalska A, Dorocka-Bobkowska B. Clinical phenotype of recurrent aphthous stomatitis and interleukin-1β genotype in a Polish cohort of patients. *J Oral Pathol Med*. 2016;1-6. https://doi.org/10.1111/jop.12533 [Epub ahead of print].
24. Ship JA, Chavez EM, Doerr PA, Henson BS, Sarmandi M. Recurrent aphthous stomatitis. *Quintessence Int*. 2000;31:95-112.
25. McDowell TL, Symons JA, Ploski R, Førre O, Duff GW. A genetic association between juvenile rheumatoid arthritis and a novel interleukin-1 alpha polymorphism. *Arthritis Rheum*. 1995;38:221-228.
26. di Giovine FS, Takhsh E, Blakemore AI, Duff GW. Single base polymorphism at -511 in the human interleukin-1 beta gene (IL1 beta). *Hum Mol Genet*. 1992;1:450.
27. Kornman KS, Crane A, Wang HY, et al. The interleukin-1 genotype as a severity factor in adult periodontal disease. *J Clin Periodontol*. 1997;24:72-77.
28. Tarlow JK, Blakemore AI, Lennard A, et al. Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat. *Hum Genet*. 1993;91:403-404.
29. Izakovicova Holla L, Borilova Linhartova P, Hrdlickova B, et al. Haplotypes of the IL-1 gene cluster are associated with gastroesophageal reflux disease and Barrett's esophagus. *Hum Immunol*. 2013;74:1161-1169.
30. Izakovicova Holla L, Musilova K, Vokurka J, et al. Association of interleukin-6 (IL-6) haplotypes with plaque-induced gingivitis in children. *Acta Odontol Scand* 2008;66:105-112.

How to cite this article: Izakovicova Holla L, Valova S, Borilova Linhartova P, et al. Association study of *interleukin-1* family, *interleukin-6*, and *its receptor* gene polymorphisms in patients with recurrent aphthous stomatitis. *J Oral Pathol Med*. 2017;00:1–6. https://doi.org/10.1111/jop.12594

1.3 Comment to the article "Association of the NOD-like receptor 3 (*NLRP3*) gene variability with recurrent aphthous stomatitis in the Czech population"

Slezakova S, Borilova Linhartova P, Masopustova L, Bartova J, Petanova J, Kuklinek P, Fassmann A, Dusek L, Izakovicova Holla L. Association of the NOD-like receptor 3 (*NLRP3*) gene variability with recurrent aphthous stomatitis in the Czech population. J Oral Pathol Med. 2018;47(4):434-439. DOI: 10.1111/jop.12694. (IF 2.237; DENTISTRY, ORAL SURGERY & MEDICINE Q1, PATHOLOGY Q2)

Author contribution: P.B.L. designed the study and genetic analysis, and participated in the writing of the manuscript.

One of the candidate genes for RAS encodes pattern recognition NLRP3, also known as NALP3 and cryopyrin. This protein is part of the NLRP3 inflammasome, a multi-protein complex which triggers the activation of inflammatory caspase 1 (Jo et al., 2016). The function of caspase 1 is to convert the pro-inflammatory cytokine precursors pro-IL-1β and pro-IL-18 into their secreted mature and active form. Caspase 1 also triggers pyroptosis, an inflammatory cell death (Aachoui et al., 2013). Independently of inflammasomes, NLRP3 acts as a transcriptional regulator of T helper (Th) 2 differentiation through binding to *IL4* promoter in $CD4^+$ Th cells of mouse model (Bruchard et al., 2015).

Alterations in the *NLRP3* gene have been associated with different multifactorial diseases with an inflammatory background and the presence of oral ulcers as one of the symptoms such as BD (Koné-Paut et al., 2007; Yüksel et al., 2014). *NLRP3* rs3806265 was previously associated with RAS development in the Iranian population (Bidoki et al., 2016).

The aim of our study was to investigate *NLRP3* rs3806265, *NLRP3* rs4612666, *NLRP3* rs10754558 SNPs both in patients with RAS, and healthy controls in the Czech population. A total of 207 subjects (64 patients with RAS and 143 healthy controls) were included in this case-control study.

The allele and genotype frequencies of *NLRP3* rs10754558 and *NLRP3* rs3806265 variants between both groups were similar. However, statistically significant differences in *NLRP3* rs4612666 genotypes between the patients with RAS and the controls were found; carriers of the TT genotype had a higher risk of developing RAS than subjects with the CT+CC genotypes (OR=14.69, 95%CI=1.73-124.72, P=0.004, $P_{corr}<0.05$). No associations between *NLRP3* haplotypes and RAS were observed.

The discrepancy between our results and the findings in the Iranian study may be due to the distinct *NLRP3* rs3806265 and rs4612666 MAF in our populations (26% vs. 14% and 16% vs. 33%, respectively, Bidoki et al., 2016).

We speculate that it is not the activity of NLRP3 inflammasome but the transcriptional role of NLRP3 in Th2 differentiation which may be engaged in the susceptibility of *NLRP3* rs4612666 TT homozygotes to RAS. Therefore, a possible lower *NLRP3* expression in TT carriers may have an impact on $CD4^+$ Th1/Th2 balance (Fig. 7). This hypothesis is in line with observations that the number of Th2 cells secreting cytokine IL-4 was decreased in patients with RAS compared with the controls (Albanidou-Farmaki et al., 2007).

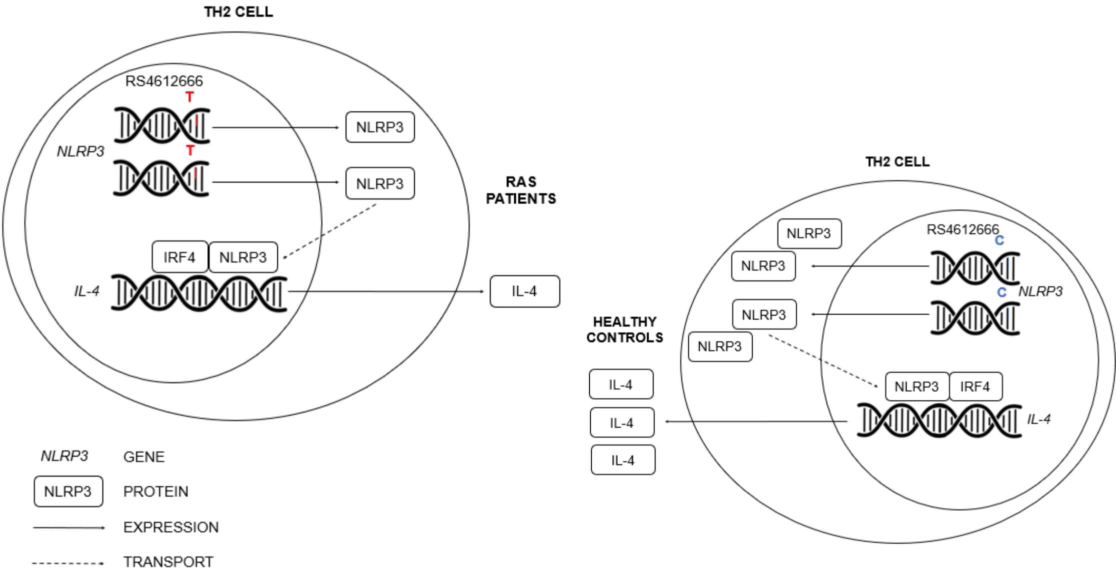


Fig. 7. Suggested role of the *NLRP3* rs4612666 SNP in the pathogenesis of RAS (Slezáková et al., 2018).

Note: The C allele of the *NLRP3* rs4612666 SNP showed a higher transcriptional enhancer activity of *NLRP3* expression in comparison to the T allele in THP-1 cells *in vitro* (Hitomi et al., 2009).

Our study indicates that the *NLRP3* rs4612666 polymorphism may be involved in the development of RAS in the Czech population.

Note: This study was presented by my student (Lucie Masopustová) at the Student scientific conference 2017 in Brno and was awarded 3rd place.

Accepted: 5 February 2018
DOI: 10.1111/jop.12694

ORIGINAL ARTICLE

WILEY

Association of the NOD-like receptor 3 (*NLRP3*) gene variability with recurrent aphthous stomatitis in the Czech population

Simona Slezakova[1,2] | Petra Borilova Linhartova[1,2] | Lucie Masopustova[3] | Jirina Bartova[4] | Jitka Petanova[5] | Pavel Kuklinek[6] | Antonin Fassmann[1] | Ladislav Dusek[7] | Lydie Izakovicova Holla[1,2]

[1]Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic
[2]Clinic of Stomatology, Institution Shared with St. Anne's Faculty Hospital and Faculty of Medicine, Masaryk University, Brno, Czech Republic
[3]Faculty of Science, Masaryk University, Brno, Czech Republic
[4]Institute of Clinical and Experimental Dental Medicine, General University Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic
[5]Department of Immunology and Microbiology, General University Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic
[6]Department of Clinical Immunology and Allergology, Institution Shared with St. Anne's Faculty Hospital and Faculty of Medicine, Masaryk University, Brno, Czech Republic
[7]Institute of Biostatistics and Analyses, Masaryk University, Brno, Czech Republic

Correspondence
Lydie Izakovicova Holla, Clinic of Stomatology and Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.
Email: holla@med.muni.cz

Background: Recurrent aphthous stomatitis (RAS) is a multifactorial disease with unclear etiopathogenesis in which disturbance of immunological processes may be involved. The aim of our study was to investigate three single nucleotide polymorphisms (SNPs) rs3806265, rs4612666, rs10754558 in *NOD-like receptor 3* (*NLRP3*), the gene encoding the component of inflammasome, in patients with RAS and healthy controls in the Czech population.

Methods: A total of 207 subjects were included in this case-control study. Sixty-four patients with RAS and 143 healthy controls were genotyped by a method based on polymerase chain reaction using 5′ nuclease TaqMan® assays. Detailed anamnestic, clinical, and laboratory data were obtained from all subjects.

Results: The allele and genotype frequencies of *NLRP3* polymorphisms (rs10754558 and rs3806265) between both groups were similar. However, statistically significant differences in *NLRP3* rs4612666 genotypes between the patients with RAS and controls were found; carriers of the TT genotype had a higher risk of developing RAS than subjects with the CT+CC genotypes (OR = 14.69, 95%CI = 1.73-124.72, $P = .004$, $P_{corr} < .05$). No associations between *NLRP3* haplotypes and RAS were observed.

Conclusions: Our study indicates that the *NLRP3* rs4612666 polymorphism may be involved in the development of RAS in the Czech population.

KEYWORDS
NLRP3, NOD-like receptor, polymorphism, recurrent aphthous stomatitis

1 | INTRODUCTION

Recurrent aphthous stomatitis (RAS) is a chronic multifactorial disease characterized by the presence of single or multiple painful erosions or ulcers on the oral mucosa. The etiopathogenesis of RAS has not been fully elucidated yet, it seems that besides stress, allergy, local trauma, microbial environment, and nutritional factors, also genetically influenced dysregulation of the immune system may play a role.[1,2]

One of the candidate genes for RAS encodes pattern recognition NOD-like receptor 3 (NLRP3, also known as NALP3 and cryopyrin). NLRP3 together with the adaptor protein (apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain, ASC) and pro-caspase 1 are part of the NLRP3 inflammasome, a multi-protein complex triggers the activation of inflammatory caspase 1.[3] Function of caspase 1 is to convert the pro-inflammatory cytokine precursors pro-interleukin (IL)-1β and pro-IL-18 into their secreted mature and active form. Caspase 1 also

 © 2018 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

triggers pyroptosis, an inflammatory cell death.[4] Independently of inflammasomes, NLRP3 acts as a transcriptional regulator of T helper (Th) 2 differentiation through binding to *Il4* promoter in $CD4^+$ Th cells of mouse model.[5]

Alterations in the *NLRP3* gene have been associated with different multifactorial diseases with inflammatory background and the presence of oral ulcers as one of the symptoms, such as Behçet's disease (BD),[6,7] Crohn's disease,[8] and celiac disease.[9,10] Recent case-control study in Iranian population suggested that single nucleotide polymorphism (SNP) *NLRP3* rs3806265 might affect RAS development.[11] The aim of our study was to investigate distribution of three *NLRP3* SNPs (rs3806265, rs4612666, rs10754558) and their influence on RAS susceptibility in the Czech population.

2 | MATERIALS AND METHODS

2.1 | Subjects and clinical examinations

Two hundred and seven Czech subjects were enrolled in this study: 64 patients with RAS (23 men and 41 women; mean age ± standard deviation [SD]: 40.2 ± 15.0 years) and 143 healthy controls (67 men and 76 women; mean age ± SD: 45.6 ± 12.5 years).

Dentists experienced in mucous membrane diagnosis and treatment made the diagnosis of RAS based on the generally accepted criteria using clinical examination and anamnestic data.[12] RAS was divided into three clinical subtypes: (i) major (larger than 1 cm and deeper than the minor form, healing within 10-30 days), (ii) minor (less than 1 cm in diameter and healing within 10-14 days), (iii) herpetiform aphthae (grouped aphthae, 1-2 mm in size) according to Karakus et al.[13] To exclude systemic disorders (such as celiac disease, Behçet's syndrome, Crohn's disease, clinical neutropenia, vitamin B12 deficiency, Erythema multiforme), the routine laboratory tests (e.g, enhanced sedimentation rate, complete blood count, hemoglobin test, white blood cell count with differential, red blood cell indices, ferritin levels, red blood cell folate assay, serum vitamin B12, basic biochemical tests—e.g, liver function tests and glucose), clinical immunologic investigation, and immunologic tests (containing e.g. ASCA IgA, ASCA IgG, and ANCA—to exclude idiopathic bowel diseases) were performed. Serological tests detecting anti-herpes simplex virus antibodies (IgM and IgG) together with clinical and anamnestic findings excluded herpetic stomatitis.

The control group was recruited from age- and sex-matched healthy individuals without history of RAS. Exclusion criteria for both groups included the presence of any other significant local or systemic diseases or pregnancy. The study protocol was approved by the Committees for Ethics of Masaryk University, Faculty of Medicine (39/2015), St. Anne's Faculty Hospital (8G/2015) and General University Hospital and First Faculty of Medicine, Charles University, Prague (53/14) and written informed consent was obtained from the study participants in line with the Declaration of Helsinki prior to their inclusion in the study.

2.2 | Genetic analysis

Genomic DNA was purified from peripheral blood leukocytes by the standard method using the phenol-chloroform extraction and proteinase K digestion of cells. Genotyping of SNPs in *NLRP3* (rs3806265, rs4612666, rs10754558) was based on polymerase chain reaction using 5′ nuclease TaqMan® assays (C__26646013_10, C__26646029_10 and C__26052028_10, respectively; Thermo Fisher Scientific, Waltham, MA, USA). The reaction mixture and conditions were designed according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany); fluorescence was measured using the LightCycler® 96 System. Application software version 1.1.0.1320 was used to analyze real-time and endpoint fluorescence data.

2.3 | Statistical analysis

Mean and SD for quantitative variables and absolute and relative frequencies for categorical variables were calculated. The chi-square test was used for comparison of the differences in the genotypes and analysis of Hardy-Weinberg equilibrium (HWE); the allele frequencies were counted from the observed numbers of genotypes by Fisher exact test. The program SNP Analyzer 2 (available on http://snp.istech21.com/snpanalyzer/2.0/) was used to determine haplotype frequencies. Odds ratio (OR), confidence intervals (CI), and *P*

TABLE 1 Clinical characteristics of the examined subjects

Clinical parameters	Controls N = 143	RAS patients N = 64
Mean age (years ± SD)	45.6 ± 12.5	40.2 ± 15.0
Sex [N of men/women (%)]	67/76 (46.9/53.1)	23/41 (35.9/64.1)
Type of aphthae		
Minor [N (%)]	NA	61 (95.3)
Major [N (%)]	NA	3 (4.7)
Herpetiform [N (%)]	NA	0 (0.0)
Mean number of lesions in each episode		
<3 lesions [N (%)]	NA	22 (34.4)
≥3 lesions [N (%)]	NA	42 (65.6)
Duration of lesions to healing		
Less than 1 week [N (%)]	NA	31 (48.4)
Less than 2 weeks [N (%)]	NA	17 (26.6)
Two or more weeks [N (%)]	NA	16 (25.0)
Number of oral ulcer recurrences		
Less than 3 times per year/irregularly [N (%)]	NA	1/4 (1.6/6.3)
At least one per 3 months [N (%)]	NA	14 (21.9)
At least one per month [N (%)]	NA	13 (20.3)
At least 2 times per month [N (%)]	NA	10 (15.6)
Permanently [N (%)]	NA	22 (34.4)

N, number of subjects; RAS, recurrent aphthous stomatitis; SD, standard deviation; NA, not applicable.

TABLE 2 *NLRP3* polymorphisms allele and genotype frequencies in patients with RAS and controls

Genotypes alleles	Controls N = 143 (%)	RAS N = 64 (%)	P	P_{corr}	OR (95%CI)
NLRP3 (rs3806265)					
CC	7 (4.9)	8 (12.5)			3.02 (1.01-9.04)
CT	54 (37.8)	25 (39.41)	.123		1.22 (0.65-2.30)
TT	82 (57.3)	31 (48.4)			1.00
C allele	68 (23.8)	41 (32.0)	.051	.459	1.51 (0.95-2.39)
T allele	218 (76.2)	87 (68.0)			1.00
NLRP3 (rs4612666)					
CC	101 (70.6)	46 (71.9)			1.00
CT	41 (28.7)	12 (18.8)	.003	.027*	0.64 (0.31-1.34)
TT	1 (0.7)	6 (9.4)			13.17 (1.54-112.59)
TT vs. CC+CT	(0.7 vs. 99.3)	(9.4 vs. 90.7)	.004	.036*	14.69 (1.73-124.72)
C allele	243 (85.0)	104 (81.3)			1.00
T allele	43 (15.0)	24 (18.8)	.209		1.30 (0.75-2.26)
NLRP3 (rs10754558)					
CC	61 (42.6)	19 (29.7)			1.00
CG	55 (38.5)	37 (57.8)	.030	.270	2.16 (1.11-4.19)
GG	27 (18.9)	8 (12.5)			0.95 (0.37-2.44)
C allele	177 (61.9)	75 (58.6)	.299		1.00
G allele	109 (38.1)	53 (41.4)			1.15 (0.75-1.76)

CI, confidence interval; N, number of subjects; NLRP3, NOD-like receptor 3; OR, odds ratio; RAS, recurrent aphthous stomatitis, *P_{corr} value < .05.

TABLE 3 Haplotype frequencies of the *NLRP3* polymorphisms in the controls and RAS group

			Controls N = 143 (%)	RAS patients N = 64 (%)	OR (95%CI)
NLRP3 (rs3806265)	*NLRP3* (rs4612666)	*NLRP3* (rs10754558)			
C	C	T	41.3	38.8	0.76 (0.49-1.18)
C	G	T	32.0	29.2	1.02 (0.66-1.58)
C	C	C	10.2	8.9	1.13 (0.59-2.19)
T	C	C	8.0	10.9	1.47 (0.77-2.80)
T	G	C	4.0	7.8	1.78 (0.65-4.89)
T	C	T	2.4	0.0	*
C	G	C	1.5	4.4	2.25 (0.31-16.2)
T	G	T	0.6	0.0	*

CI, confidence interval; N, number of subjects; NLRP3, NOD-like receptor 3; OR, odds ratio; RAS, recurrent aphthous stomatitis.
Haplotypes are ordered according to the decreasing haplotype frequency in the healthy controls.
*Cannot be assessed due to a small number.

values were calculated. The Bonferroni correction was used to adjust the level according to the number of independent comparisons (P_{corr}). Power analysis was conducted with respect to case-control design of the study. All statistical analyses were performed using the program package Statistica v. 12 (StatSoft Inc., Tulsa, Okla., USA) and SPSS software (SPSS 22, IBM Corporation, 2013).

3 | RESULTS

The studied group included 64 patients with RAS and 143 unrelated healthy control subjects matched by sex and age. Most of the patients with RAS (95.3%) suffered from minor aphthae; no subjects had a herpetiform type of this disease. More than 90% of RAS patients recruited had at least four recurrences of oral erosions/ulcers per year (Table 1) and in 28 patients (43.8%) aphthae were diffused (occurring in three and more sites in the oral mucosa). All the immunological and serological tests conducted in the group of patients with aphthous stomatitis ($N = 64$) were negative. However, these patients were recruited from a bigger set of patients with RAS who had been examined at the departments of immunology in St. Anne's Faculty Hospital in Brno or General University Hospital in Prague. Patients with blood test alterations or different clinical symptoms were excluded from subsequent genetic analysis (e.g,

FIGURE 1 Suggested role of the *NLRP3* polymorphism rs4612666 in the pathogenesis of RAS. The C allele of the *NLRP3* polymorphism rs4612666 showed a higher transcriptional enhancer activity of *NLRP3* expression in comparison with the T allele in THP-1 cells *in vitro*.[16] Although NLRP3 is best appreciated for its innate immunological role mediating inflammasome activation, now NLRP3 debuts as a transcription factor key for Th2 polarization.[20] In Th2 cells, NLRP3 is trafficked to the nucleus and there, together with transcriptional activator interferon regulatory factor 4 (IRF4), it binds to regulatory DNA sites and promotes the transcription of gene encoding IL-4.[5] Findings regarding decreased IL-4 Th2 cells secretion in patients with RAS compared with the controls[18] and also significant association of the TT genotype *NLRP3* rs4612666 with a higher risk for RAS development in comparison with CC+CT genotypes in our study are in line with this molecular hypothesis. We assume that the lower *NLRP3* expression in TT carriers may have an impact on CD4+ Th1/Th2 balance affecting thus the pathogenesis of RAS [Colour figure can be viewed at wileyonlinelibrary.com]

those with higher levels of antibodies against herpes simplex virus, with anemia, or with suspected autoimmune bullous dermatoses). None of the patients suffered from inflammatory bowel disease or any other immunological disorders, and none received any systemic treatment that could influence the results of the tests performed in this study.

The power calculation was based on controls: RAS ratio of approx. 2.2 and estimated statistically detectable difference ($\alpha < 0.05$, $\beta = 0.20$) $\pm$ 16% for the total comparison of alleles. The sample size enrolled is thus sufficient for the results reached in case of SNP rs4612666. The differences detected in the other two comparisons were not presented as statistically significant and numerically they reached <10% range, which is quite a small difference to be generalized. The allele and genotype distribution in *NLRP3* is presented in Table 2. The frequencies of the studied SNPs were in compliance with those expected by the HWE in the controls, with the exception of the *NLRP3* polymorphism (rs10754558) deviating from the HWE ($P < .05$).

Although the allele and genotype frequencies of the *NLRP3* polymorphisms (rs3806265 and rs10754558) between the groups of patients with RAS and healthy controls did not differ, statistically significant differences in the *NLRP3* (rs4612666) genotype distributions were found ($P = .003$, $P_{corr} < .05$). The comparison of the TT versus CT+TT genotype frequencies between the patients with RAS and healthy controls revealed significant differences, carriers of the TT genotype had a higher risk of RAS development (OR = 14.69, 95% CI = 1.73-124.72, $P = .004$, $P_{corr} < .05$). The analysis of three *NLRP3* SNPs revealed eight haplotypes in the studied subjects. However, no significant differences among the *NLRP3* haplotypes in healthy controls and patients with RAS were found ($P > .05$, Table 3).

4 | DISCUSSION

Although the etiopathogenesis of RAS is unclear, it is considered a multifactorial disease with both environmental and genetic contributions. Studies implied the involvement of Th1-type immune response in the formation of aphthous ulcers as the Th1 related genes were overexpressed and on the contrary, the Th2 related genes were downregulated in RAS tissues compared to healthy mucosa.[14,15] Disproportion of the $CD4^+$ Th1/Th2 immune response and consequently the discrepancy in pro- and anti-inflammatory cytokine production may be involved in a loss of the immune tolerance in the oral mucosa leading to an inflammatory reaction and development of ulcers.

The *NLRP3* gene variability has recently been identified as a susceptibility factor for RAS in the Iranian population.[11] In agreement with the results of this study, we did not observe correlation between SNP *NLRP3* rs10754558 and RAS, even though this variant

seems to influence *NLRP3* mRNA stability.[16] In contrast, we did not detect any significant association between *NLRP3* rs3806265 and RAS.[11] Furthermore, unlike the Iranian study, the *NLRP3* polymorphism (rs35829419) was excluded from our analysis as its frequency in the Caucasian population is low.

Although Bidoki et al.[11] suggested that *NLRP3* SNP rs4612666 did not affect the RAS development in Iranians, TT homozygotes of this *NLRP3* SNP had a higher risk of developing RAS in comparison with subjects with the CT+CC genotypes in our population. The *NLRP3* rs4612666 C allele showed a higher transcriptional enhancer activity of *NLRP3* expression in comparison with the T allele in THP-1 cells *in vitro*.[16]

As the levels of IL-1 and IL-18 are higher in RAS patients in comparison with healthy controls,[17] we speculate that not the activity of NLRP3 inflammasome but the transcriptional role of NLRP3 in Th2 differentiation may be engaged in susceptibility of *NLRP3* rs4612666 TT homozygotes to RAS. Therefore, a possible lower *NLRP3* expression in TT carriers may have an impact on CD4$^+$ Th1/Th2 balance (Figure 1). This hypothesis is in line with observations that the number of Th2 cells secreting cytokine IL-4 was decreased in patients with RAS compared with the controls.[18] The proposed role of *NLRP3* SNP rs4612666 and consequently NLRP3 transcriptional function in the pathogenesis of immune-related diseases is supported by the findings that the C allele with a higher expression increases the risk of developing aspirin-induced asthma.[16] As one of the factors driving aspirin-induced asthma is IL-4[19] and moreover, the activation of NLRP3 seems to be involved in the promotion of asthma in ovalbumin-induced allergy model,[5] this association is not surprising.

Inconsistency in our and previous results may be due to the distinct *NLRP3* rs3806265 and rs4612666 minor allele frequency (MAF) in Czech and Iranian population (26% vs. 14% and 16% vs. 33%, respectively).[11] In addition, discrepancy may be explained by different approaches to data and clinical evaluation and selection of patients.

Limitations of our study are related to the case-control approach vulnerable to population stratification. However, we investigated three *NLRP3* polymorphisms with possible functional relevance and their haplotypes in exclusively Czech Caucasian individuals. The next complicating factor is that the small number of subjects enrolled, especially in the group of patients, may limit the statistical power of our study. The power calculation with standard statistical procedures applied showed that our sample size was sufficient to detect significant differences, especially for rs4612666 variant. In addition, one *NLRP3* polymorphism (rs10754558) did not comply with HWE in controls. Numerically, the deviation from HWE was caused by asymmetric distribution profile of the alleles for only this variant. Therefore, the study does not present any significant result which could be affected by the deviation from the HWE.

To our knowledge, this study represents the first evidence of association between *NLRP3* and RAS in European Caucasian population. Although the effect of *NLRP3* rs3806265 on the development of RAS was not confirmed, our findings suggest a strong association between *NLRP3* rs4612666 and RAS in the Czech population. However, further studies in larger independent cohort are required to prove our results.

ACKNOWLEDGEMENTS

This study was supported by grants AZV 15-29336A, GACR GB14-37368G, and project MUNI/A/0948/2016.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

S.V, P.B.L., J.B., J.P., and L.I.H. designed the study and drafted the paper. J.B., J.P., P.K., and A.F. performed the clinical analysis and collected the blood samples. L.M. and S.V. carried out the genetic analysis, L.I.H. and L.D. performed statistical analysis. All authors revised the final version of the manuscript.

ORCID

Lydie Izakovicova Holla http://orcid.org/0000-0002-7610-8929

REFERENCES

1. Ślebioda Z, Szponar E, Kowalska A. Recurrent aphthous stomatitis: genetic aspects of etiology. *Postepy Dermatol Alergol*. 2013;30:96-102.
2. Ślebioda Z, Szponar E, Kowalska A. Etiopathogenesis of recurrent aphthous stomatitis and the role of immunologic aspects: literature review. *Arch Immunol Ther Exp*. 2014;62:205-215.
3. Jo EK, Kim JK, Shin DM, Sasakawa C. Molecular mechanisms regulating NLRP3 inflammasome activation. *Cell Mol Immunol*. 2016;13:148-159.
4. Aachoui Y, Sagulenko V, Miao EA, Stacey KJ. Inflammasome-mediated pyroptotic and apoptotic cell death, and defense against infection. *Curr Opin Microbiol*. 2013;16:319-326.
5. Bruchard M, Rebé C, Derangère V, et al. The receptor NLRP3 is a transcriptional regulator of TH2 differentiation. *Nat Immunol*. 2015;16:859-870.
6. Yüksel Ş, Eren E, Hatemi G, et al. Novel NLRP3/cryopyrin mutations and pro-inflammatory cytokine profiles in Behçet's syndrome patients. *Int Immunol*. 2014;26:71-81.
7. Koné-Paut I, Sanchez E, LeQuellec A, Manna R, Touitou I. Autoinflammatory gene mutations in Behçet's disease. *Ann Rheum Dis*. 2007;66:832-834.
8. Villani A-C, Lemire M, Fortin G, et al. Common variants in the NLRP3 region contribute to Crohn's disease susceptibility. *Nat Genet*. 2009;41:71-76.
9. Pontillo A, Vendramin A, Catamo E, Fabris A, Crovella S. The missense variation Q705K in CIAS1/NALP3/NLRP3 gene and an NLRP1 haplotype are associated with celiac disease. *Am J Gastroenterol*. 2011;106:539-544.
10. Pontillo A, Brandao L, Guimaraes R, Segat L, Araujo J, Crovella S. Two SNPs in NLRP3 gene are involved in the predisposition to type-1 diabetes and celiac disease in a pediatric population from northeast Brazil. *Autoimmunity*. 2010;43:583-589.
11. Bidoki AZ, Harsini S, Sadr M, et al. NLRP3 gene polymorphisms in Iranian patients with recurrent aphthous stomatitis. *J Oral Pathol Med*. 2016;45:136-140.
12. Ship JA, Chavez EM, Doerr PA, Henson BS, Sarmadi M. Recurrent aphthous stomatitis. *Quintessence Int*. 2000;31:95-112.

13. Karakus N, Yigit S, Rustemoglu A, Kalkan G, Bozkurt N. Effects of interleukin (IL)-6 gene polymorphisms on recurrent aphthous stomatitis. *Arch Dermatol Res*. 2014;306:173-180.
14. Borra RC, Andrade PM, Silva IDCG, et al. The Th1/Th2 immune-type response of the recurrent aphthous ulceration analyzed by cDNA microarray. *J Oral Pathol Med*. 2004;33:140-146.
15. Lewkowicz N, Kur B, Kurnatowska A, Tchorzewski H, Lewkowicz P. Expression of Th1/Th2/Th3/Th17-related genes in recurrent aphthous ulcers. *Arch Immunol Ther Exp*. 2011;59:399-406.
16. Hitomi Y, Ebisawa M, Tomikawa M, et al. Associations of functional NLRP3 polymorphisms with susceptibility to food-induced anaphylaxis and aspirin-induced asthma. *J Allergy Clin Immunol*. 2009;124:779-785.
17. Ozyurt K, Çelik A, Sayarlıoglu M, et al. Serum Th1, Th2 and Th17 cytokine profiles and alpha-enolase levels in recurrent aphthous stomatitis. *J Oral Pathol Med*. 2014;43:691-695.
18. Albanidou-Farmaki E, Markopoulos AK, Kalogerakou F, Antoniades DZ. Detection, enumeration and characterization of T helper cells secreting type 1 and type 2 cytokines in patients with recurrent aphthous stomatitis. *Tohoku J Exp Med*. 2007;212:101-105.
19. Steinke JW, Borish L. Factors driving the aspirin exacerbated respiratory disease phenotype. *Am J Rhinol Allergy*. 2015;29:35-40.
20. Ting JPY, Harton JA. NLRP3 moonlights in TH2 polarization. *Nat Immunol*. 2015;16:794-796.

How to cite this article: Slezakova S, Borilova Linhartova P, Masopustova L, et al. Association of the NOD-like receptor 3 (*NLRP3*) gene variability with recurrent aphthous stomatitis in the Czech population. *J Oral Pathol Med*. 2018;47:434-439. https://doi.org/10.1111/jop.12694

2. External apical root resorption (EARR)

Note: This chapter builds on my Dissertation thesis (Bořilová Linhartová, 2014).

EARR is a relatively frequent adverse consequence of treatment with a fixed orthodontic appliance caused by the activation of odontoclasts which results in a loss of dental tissues. It is a pathological process manifested by an irreversible shortening of the tooth's root.
EARR is assumed to have a multifactorial etiopathogenesis where biological and mechanical effects are involved in its development and progression.
At the molecular level, regulatory proteins modulating the inflammation reaction (adenosine triphosphate – ATP/ purinergic receptor – P2RX7/ IL-1β) and controlling the activation of osteoclasts (nucleic factor receptor activator kB – RANK/ ligand activator of nuclear factor kB – RANKL/ osteoprotegerin – OPG) are considered as key factors. Osteopontin (OPN), also known as secreted phosphoprotein 1 (SPP1), is one of the noncollagenous proteins present in the bone matrix. It is also produced by activated cells of the immune system. OPN plays a critical role in the maintenance of bone, especially as a molecule involved in the response of bones to external stress. It is also involved in other homeostatic defense mechanisms within the mammalian organism. The role of OPN in inflammatory conditions in the oral cavity is still poorly understood (Rittling and Singh, 2015).
Based on the etiopathogentic hypothesis, genes involved in the modulation of inflammatory response and bone remodeling are studied in patients with postorthodontic EARR. Findings observed in our studies in the Czech population (Linhartova et al., 2013; Borilova Linhartova et al., 2017) were included in the work from Hartsfield et al. (2017) and the overview figure was created (Fig. 8).

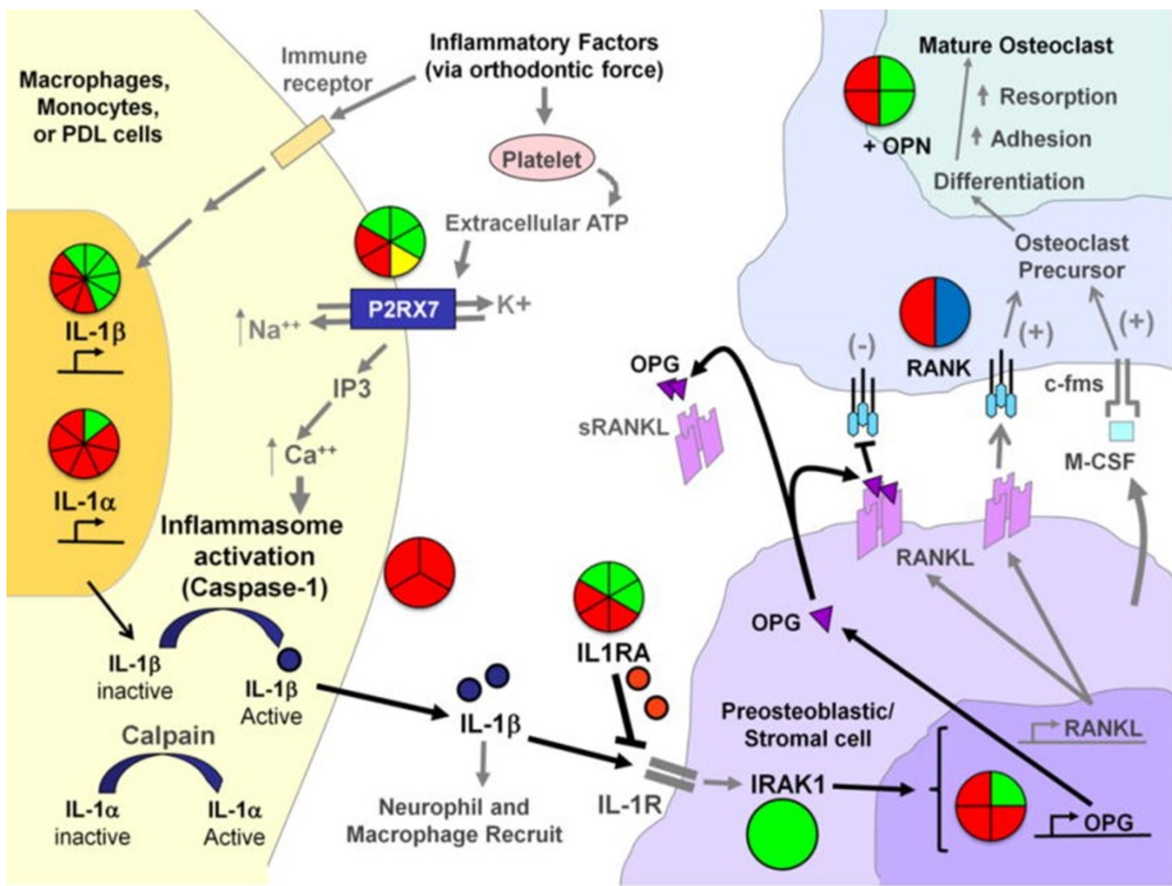


Fig. 8. Genetic associations to EARR within the ATP/P2RX7/IL-1β and RANK/RANKL/OPG signaling pathways. The pie-charts located throughout the diagram summarize the genetic association and linkage findings connected to different factors within the pathways and EARR. The number of triangles within each pie shape summarize the number of markers or independent tests that were examined for each factor (Hartsfield et al., 2017).

green/red/yellow/blue indicates genetic association/no association/haplotype association/genetic linkage

2.1 Comment to the article "Genetic determinants and postorthodontic external apical root resorption in Czech children"

Borilova Linhartova P, Cernochova P, Kastovsky J, Vrankova Z, Sirotkova M, Izakovicova Holla L. Genetic determinants and postorthodontic external apical root resorption in Czech children. Oral Dis. 2017;23(1):29-35. DOI: 10.1111/odi.12564. (IF 2.31; DENTISTRY, ORAL SURGERY & MEDICINE Q1)

Author contribution: P.B.L. designed the study, drafted the paper, and particularly carried out genetic analysis.

Candidate genes and their specific polymorphisms were selected on the basis of previously described associations in other populations (see Overview in the Table on the page 59), proven functional effects in the etiopathogenesis of EARR, an MAF higher than 0.1 in the population studied, and/or localization within the haplotype structure of the gene. Although IL-17 is a pro-inflammatory cytokine which plays a role in odontoclastogenesis, no *IL-17* gene variant had been studied in relation to EARR before.

The aim of this work was to analyze the possible associations between the selected clinical and treatment-related parameters, seven SNPs in the *IL-17A*, *P2RX7*, *SPP1*, and *TNFRSF11B* (encoding OPG) genes, and EARR in Czech children after orthodontic treatment.

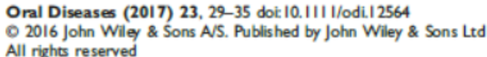
This case-control study comprised 99 orthodontically treated patients (69 controls and 30 subjects with EARR). Genotype determinations of *IL-17A* rs2275913, *P2RX7* rs208294, *P2RX7* rs1718119, *SPP1* rs11730582, *SPP1* rs9138, *TNFRSF11B* rs3102735, and *TNFRSF11B* rs2073618 were carried out.

While no significant differences were observed in the allele or genotype frequencies of all seven of the studied SNPs, the specific haplotype composed of *P2RX7* (rs208294 and rs1718119) variants modified the risk of EARR development ($P<0.05$, $OR=4.06$, $95\%CI=1.05–15.66$). In addition, the length of treatment with a fixed orthodontic appliance positively correlated with the presence of EARR ($P<0.05$).

Consistent with the conclusions of other studies in other populations (Pereira et al., 2014; Sharab et al., 2015), the CG haplotype of *P2RX7* gene was found more often in Czech patients with postorthodontic EARR than in the controls. This haplotype containing G allele *P2RX7* rs1718119 SNP, which encodes alanine in the protein position +348, predisposes to a gain of function and increased pro-inflammatory IL-1β secretion (Stokes et al., 2010; Sun et al., 2010; Diercke et al., 2012).

Although the effect of individual SNPs studied on the EARR development was not confirmed in the Czech population, complex analysis suggested that variability in the *P2RX7* gene and the length of orthodontic treatment may be important factors contributing to the etiopathogenesis of postorthodontic EARR. This association should however be considered carefully due to the small size of the studied population.

Note: This study was presented by my students (Zuzana Vranková, Martina Sirotková) at the Student scientific conference in 2016 in Brno and at the International Spring meeting in 2018 of the Italian orthodontic society in Naples, Italy and was awarded 1st place at both meetings.



Oral Diseases (2017) 23, 29–35 doi:10.1111/odi.12564
© 2016 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd
All rights reserved
www.wiley.com

ORIGINAL ARTICLE

Genetic determinants and postorthodontic external apical root resorption in Czech children

P Borilova Linhartova[1,2], P Cernochova[1], J Kastovsky[3,*], Z Vrankova[3,*], M Sirotkova[3,*], L Izakovicova Holla[1,2]

[1]*Clinic of Stomatology, Institutions Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Brno;* [2]*Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno;* [3]*Faculty of Medicine, Masaryk University, Brno, Czech Republic*

OBJECTIVE: Genes, involved in the modulation of inflammatory response and bone remodeling, play a role in the development of postorthodontic external apical root resorption (EARR). The aim of our study was to analyze possible associations between seven single nucleotide polymorphisms (SNPs) in *interleukin-17A* (*IL-17*), *osteopontin* (*SPP1*), *purinoreceptor P2X7* (*P2RX7*), and *tumor necrosis factor receptor superfamily member 11B* (*TNFRSF11B*) genes and EARR in children after orthodontic treatment.

SUBJECTS AND METHODS: This case–control study comprised 99 orthodontically treated patients (69 controls and 30 subjects with EARR). Genotype determinations of rs2275913, rs11730582, rs9138, rs208294, rs1718119, rs3102735, and rs2073618 were based on polymerase chain reaction using 5′ nuclease TaqMan® assays.

RESULTS: While no significant differences were observed in allele or genotype frequencies of all seven studied SNPs, specific haplotype of *P2RX7* (rs208294 and rs1718119) modified the risk of EARR development ($P < 0.05$). In addition, the length of treatment with a fixed orthodontic appliance positively correlated with the presence of EARR ($P < 0.05$).

CONCLUSIONS: Although the effect of individual SNPs studied on the EARR development was not confirmed in the Czech population, complex analysis suggested that variability in the *P2RX7* gene and the length of orthodontic treatment may be important factors contributing to the etiopathogenesis of postorthodontic EARR.

Oral Diseases (2017) 23, 29–35

Keywords: gene polymorphism; orthodontic treatment; root resorption

Correspondence: Lydie Izakovicova Holla, MD, PhD, Clinic of Stomatology and Department of Pathophysiology, Faculty of Medicine, Masaryk University, Pekarska 664/53, 602 00 Brno, Czech Republic. Tel: +420 543183406, Fax: +420 543183456, E-mail: holla@med.muni.cz
*Authors work equally on this paper.
Received 7 June 2016; revised 27 July 2016; accepted 29 July 2016

Introduction

External apical root resorption (EARR) is a relatively frequent adverse consequence of treatment with a fixed orthodontic appliance caused by activation of odontoclasts which results in a loss of dental tissues (i.e., cement and dentin) (Brezniak and Wasserstein, 2002; Hartsfield *et al*, 2004). Root resorption occurs already during the initial phase of therapy, the most affected teeth being the upper incisors (Smale *et al*, 2005; Kocadereli *et al*, 2011). However, the prevalence of severe EARR resulting from orthodontic treatment was low (about 3%) (Maués *et al*, 2015). EARR may also be present in a small percentage of persons who have never undergone orthodontical treatment (Harris *et al*, 1993).

Numerous factors, such as race, age, gender, malocclusion, the shape of roots, extent and direction of displacement of the upper incisors, type and length of orthodontic treatment, can participate in the EARR development (Marek *et al*, 2001; Picanço *et al*, 2013; Maués *et al*, 2015). These factors cannot fully explain the differences in susceptibility to EARR. In 1975, Newman described the familial occurrence of EARR (Newman, 1975). Harris *et al* (1997) published a hypothesis about a possible genetic impact on the EARR development using a sibling model. A retrospective twin study conducted by Ngan *et al* (2004) then found evidence for both genetic and environmental factors influencing EARR. In addition, the involvement of multiple genes in the etiopathogenesis of EARR was demonstrated in a mouse model (Abass *et al*, 2008).

During the study of hereditary risk factors, Hartsfield *et al* (2004) focused on genes for proteins involved in bone remodeling. Many studies have confirmed important roles of proteins involved in the modulation of the inflammatory response, that is, adenosine triphosphate/purinoreceptor P2X7/interleukin-1β (ATP/P2RX7/IL-1β) and regulatory proteins of osteoclast activation, that is, receptor activator of nuclear factor kappa B/its ligand/osteoprotegerin (RANK/RANKL/OPG) (Katagiri and Takahashi, 2002; Hartsfield, 2009; Quinn and Saleh, 2009).

Polymorphisms in *caspase-1* (*CASP-1*), *IL-1* gene cluster, *osteopontin* (*SPP1*, *secreted phosphoprotein 1*), *purinoreceptor P2X7* (*P2RX7*, *purinergic receptor P2X, ligand-gated ion channel, 7*), *tumor necrosis factor-alpha* (*TNF-α*), *tissue non-specific alkaline phosphatase* (*TNSALP*), *vitamin D receptor* (*VDR*), and/or genes encoding RANK/OPG in relation to EARR have been previously studied in different populations (for the literature review, see Table 1). SNP rs1143634 in the *IL-1B* gene, currently one of the most studied polymorphism, was the subject of a meta-analysis by Wu *et al* (2013). Although the family-based study by Al-Qawasmi *et al* (2003b) reported relation between *TNF superfamily member 11A* (*TNFRSF11A*) locus encoding RANK and EARR development, only a few single nucleotide or tandem repeat variants (SNPs or VNTRs) in other candidate genes have been associated with postorthodontic EARR, for example, *IL-1A* (Gülden *et al*, 2009), *IL-1 receptor antagonist* (*IL-1RN*) (Iglesias-Linares *et al*, 2013; Linhartova *et al*, 2013), *P2RX7* (Pereira *et al*, 2014; Sharab *et al*, 2015), *SPP1* (Iglesias-Linares *et al*, 2014), and *TNFRSF11B* encoding osteoprotegerin (Hartsfield, 2009).

In addition, Hayashi *et al* (2012) suggested that Th17 cells may aggravate the process of orthodontically induced inflammatory root resorption, as the proinflammatory interleukin-17A (IL-17A) stimulated odontoclastogenesis and influenced the mRNA expression of RANKL from human dental pulp cells *in vitro* (Nakano *et al*, 2015). No study has investigated *IL-17* gene variability in postorthodontic EARR till now. Recently, the *IL-17A* -197A/G (rs2275913) functional polymorphism has been associated with IL-17 production in mononuclear cells and the 'red complex' bacteria occurrence in Czech non-diabetic and diabetic (type 1) patients suffering from chronic periodontitis (Borilova Linhartova *et al*, 2016).

The aim of this work was to analyze possible associations between the selected clinical and treatment-related parameters, seven SNPs in the *IL-17A*, *P2RX7*, *SPP1*, and *TNFRSF11B* genes, and EARR in Czech children after orthodontic treatment. Candidate genes and their specific polymorphisms were selected on the basis of previously described associations in other populations (see Table 1), proven functional effects in etiopathogenesis of EARR, minor allele frequency (MAF) higher than 0.1 in the population studied, and/or localization within the haplotype structure of the gene.

Materials and methods

Subjects and clinical examinations

For this case–control retrospective study, 99 Caucasian children of Czech origin [37 boys and 62 girls, 15.0 ± 4.7 (mean age ± standard deviation)] were selected from the patients pool of the Orthodontic Department, Clinic of Stomatology, Institutions Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Brno, Czech Republic, from 2005 to 2015, and demographic data are shown in Table 2. All subjects had the complete anamnestical and clinical data available, and clearly assessable X-ray radiographs (orthopantomograms and lateral cephalometric radiographs) taken before and after treatment using the same machine. The exclusion criteria were previous injury of teeth, endodontic or earlier fixed orthodontic appliance therapy, and the presence of systemic disease affecting the hard tissues. The inclusion criteria contained the presence of maxillary incisors free of fractures or abrasion on the incisal edges between measurements before and after orthodontic treatment and agreement to participate in the study. All the patients were treated by one physician (P.C.), who decided on the EARR diagnosis according to Al-Qawasmi *et al* (2003a,b). Differences in root and crown lengths of maxillary incisors were determined on the basis of the measurement procedure described in our previous study (Linhartova *et al*, 2013).

Written informed consent was obtained from all participants in line with the Helsinki Declaration before inclusion in the study, which was performed with the approval of the Committees for Ethics of the Medical Faculty, Masaryk University Brno and St. Anne's Faculty Hospital.

Genetic analysis

Isolation and storage of DNA and genotyping of samples were conducted in the laboratory of the Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.

Genotyping of seven SNPs in *IL-17A* -197A/G (rs2275913), *P2RX7* +489C/T (rs208294, Tyr155His) and +1068C/T (rs1718119, Thr348Ala), *SPP1* -443T/C (rs11730582) and +1239A/C (rs9138), *TNFRSF11B* -163C/T (rs3102735) and +1181C/G (rs2073618, Lys3Asn) was based on polymerase chain reaction using 5′ nuclease TaqMan® assays (see Table 3). Reaction mixture and conditions were designed according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA), fluorescence was measured using the ABI PRISM 7000 Sequence Detection System, and SDS version 1.2.3 software was used to analyze real-time and endpoint fluorescence data. Genotyping was performed by P.B.L, J.K., Z.V., and M.S. unaware of the phenotype and 10% of samples were analyzed in duplicates.

Statistical analysis

Statistical analysis was performed using the standard descriptive statistics, and absolute and relative frequencies for categorical variables and mean with standard deviation (s.d.) for continuous variables were determined. Allele frequencies were calculated from the observed number of genotypes. Fisher's exact test was used to assess the significance of the differences between the groups of patients and controls. Significance of deviations from Hardy–Weinberg equilibrium and differences in the genotype frequencies for each polymorphism were calculated using the chi-square test. Linkage disequilibrium was measured by Lewontin standardized disequilibrium coefficient (D') and haplotypes were determined using the program SNP Analyzer 2 (available on http://snp.istech.info/istech/board/login_form.jsp). Odds ratio (OR), confidential intervals (CI), and levels of significance (P) were calculated using the software package Statistica v. 12.0 (Statsoft Inc., Tulsa, OK, USA).

Results

The studied cohort included 30 patients with EARR (11 boys and 19 girls, mean age 14.6 ± 3.2 years) and 69 unrelated healthy control subjects comparable by sex and age (26 boys and 43 girls, mean age 15.2 ± 5.3 years) in the retention phase after fixed orthodontic appliance therapy. There were no significant differences in the representation of classes according to Angle's classification or type of treatment (extraction/non-extraction) between the EARR patients and controls. Of the clinical variables, only duration of treatment showed significant differences between cases and controls ($P < 0.05$) (see Table 2).

Genotype frequencies of all seven studied SNPs were in Hardy–Weinberg equilibrium ($P > 0.05$). Distribution of alleles and genotypes in selected loci of *IL-17A*, *P2RX7*, *SPP1*, and *TNFRSF11B* genes was similar between the control and EARR groups (see Table 4). Haplotype CG composed of *P2RX7* +489C/T (rs208294, Tyr155His) and +1068G/A (rs1718119, Thr348Ala) variants was found significantly more frequently in EARR patients than in controls (in recessive model: $P < 0.05$, OR = 4.06, 95%

Table 1 Review of studied polymorphisms in EARR-association and linkage studies

Gene	*Polymorphism (rs number)*	*Paper*	*Study design*	N *(controls/EARR patients)*	*Race/(Nation)*	*Association with EARR*
CASP1	rs530537 rs580253 rs554344	Sharab *et al* (2015)	Case–control	134 (67/67)	Caucasian (USA)	NS
IL-1	*IL-1B* rs1143634	Pereira *et al* (2014)	Retrospective DV: %EARRmax	195	Caucasian (Portugal)	NS
		Tomoyasu *et al* (2009)	Case–control	54 (24/27)	Asian (Japan)	NS
		Bastos Lages *et al* (2009)	Case–control	61 (38/23)	Mixed (Brazil)	Risk genotype TT
	IL-1A rs180058 *IL-1B*	Gülden *et al* (2009)	Case–control	(49/45)	Caucasian (German)	*IL-1A*: Risk genotype TT *IL-1B*: NS
	rs1143634	Al-Qawasmi *et al* (2003a)	Retrospective family study DV: %EARRmax	118	Caucasian (USA)	*IL-1A*: NS *IL-1B*: Risk genotype CC
	IL-1A rs180058 *IL-1B*	Sharab *et al* (2015)	Case–control	134 (67/67)	Caucasian (USA)	NS
	rs1143634 *IL-1RN* rs419598	Bastos *et al* (2015)	Retrospective DV: EARR indexes	146 after replantation of permanent teeth	Mixed (Brazil)	NS
		Iglesias-Linares *et al* (2012) Iglesias-Linares *et al* (2013)	Case–control	93 (54/39)	Caucasian (Spain)	*IL-1A*: NS *IL-1B*: Risk genotype TT *IL-1RN*: Risk genotype TT
	or 86 bp VNTR	Linhartova *et al* (2013)	Case–control	106 (74/32)	Caucasian (Czech)	*IL-1A*: NS *IL-1B*: NS *IL-1RN*: Risk genotype *IL-1RN**22 NS in haplotype
P2RX7	rs208294	Sharab *et al* (2015)	Case–control	134 (67/67)	Caucasian (USA)	Risk genotypes CC or CT
	rs1718119	Sharab *et al* (2015)	Case–control	134 (67/67)	Caucasian (USA)	NS
		Pereira *et al* (2014)	Retrospective DV: %EARRmax	195	Caucasian (Portugal)	Risk genotype GG
	rs2230912	Sharab *et al* (2015)	Case–control	134 (67/67)	Caucasian (USA)	NS
SPP1	rs9138	Iglesias-Linares *et al* (2014)	Case–control	87 (50/37)	Caucasian (Spain)	Risk genotype CC
	rs11730582	Iglesias-Linares *et al* (2014)	Case–control	87 (50/37)	Caucasian (Spain)	Risk genotype CC NS in haplotype
TNF-α	rs1800629	Al-Qawasmi *et al* (2003b)	Retrospective family study DV: EARR phenotype	124	Caucasian (USA)	NS
TNFRSF11A	rs1805034	Pereira *et al* (2014)	Retrospective DV: %EARRmax	195	Caucasian (Portugal)	NS
	Microsatellite Marker D18S64	Al-Qawasmi *et al* (2003b)	Retrospective family study DV: EARR phenotype	124	Caucasian (USA)	Associated with EARR
TNFRSF11B	rs3102735	Pereira *et al* (2014)	Retrospective DV: %EARRmax	195	Caucasian (Portugal)	NS
	rs2073618	Hartsfield (2009)	Case–control	135	Caucasian (USA)	Risk genotype CC
TNSALP	AL215L	Al-Qawasmi *et al* (2003b)	Retrospective family study DV: EARR phenotype	124	Caucasian (USA)	NS
VDR	rs731236	Fontana *et al* (2012)	Case–control	377 (160/179 and 38 untreated)	Mixed (Brazil)	NS (comparison between groups of treated patients)

CASP-1, caspase-1; DV, dependent variable; EARR, external apical root resorption; IL-1A (-B, -RN), interleukin-α (-β, -receptor antagonist); NS, non-significant differences; P2RX7, purinoreceptor P2X7; SPP1, secreted phosphoprotein 1 or osteopontin; TNF-α, tumor necrosis factor-α; TNFRSF11A, tumor necrosis factor receptor superfamily member 11A or receptor activator of nuclear factor kappa B; TNFRSF11B, tumor necrosis factor receptor superfamily member 11B or osteoprotegerin; TNSALP, tissue non-specific alkaline phosphatase; VDR, vitamin D receptor.

CI = 1.05–15.66 without correction for multiple comparisons) (see Table 5). No association between *SPP1* or *TNFRSF11B* haplotypes and postorthodontic EARR was found (data not shown).

Discussion

The etiopathogenesis of EARR is considered to be multifactorial, and it is affected by several external and internal

Table 2 The clinical and treatment-related characteristics of examined subjects

Clinical parameters	*Controls* N = *69*	*EARR patients* N = *30*
Mean age (years ± s.d.)	15.2 ± 5.3	14.6 ± 3.2
Sex [N (%)]		
Boys	26 (37.7)	11 (36.7)
Girls	43 (62.3)	19 (63.3)
Angle's classification [N (%)]		
Class I	29 (42.0)	13 (43.3)
Class II	33 (47.8)	14 (46.7)
Class III	7 (10.2)	3 (10.0)
Treatment [N (%)]		
Extraction	22 (31.9)	12 (40.0)
Non-extraction	47 (68.1)	18 (60.0)
Duration of treatment* [N (%)]		
<22 months	15 (21.7)	3 (10.0)
22–27 months	11 (15.9)	7 (23.3)
27–34 months	22 (31.9)	4 (13.3)
>34 months	21 (30.5)	16 (53.4)

EARR, external apical root resorption; N, number of subjects; s.d., standard deviation.
**P*-value < 0.05.

factors. In line with results of previous studies in other populations (Segal *et al*, 2004; Sharab *et al*, 2015), a longer length of treatment with fixed orthodontical appliance positively correlated with the presence of EARR in Czech children. No other clinical factors studied (Angle's classification, type of treatment—extraction *vs* non-extraction) or gender affected the EARR development in our set.

On the molecular level, there are two main signaling pathways involved in the development of inflammatory EARR (Hartsfield, 2009). The ATP/P2RX7/IL-1β inflammation pathway plays a role in the activation control of osteoclasts (Grol *et al*, 2009; Kvist *et al*, 2014) and the RANK/RANKL/OPG system is involved in the alveolar remodeling and physiologically or orthodontically induced root resorption (Tyrovola *et al*, 2008).

In this study, for the first time, we analyzed the functional SNP in the *IL-17* gene in relation to EARR. Although pro-inflammatory IL-17 plays a role in odontoclastogenesis, polymorphism *IL-17A* -197A/G (rs2275913) was not associated with postorthodontic EARR in Czech children. Besides *IL-1* gene variants, associations between EARR and SNPs in genes encoding OPG (Hartsfield, 2009), P2RX7 (Pereira *et al*, 2014; Sharab *et al*, 2015), and SPP1 (Iglesias-Linares *et al*, 2014) were found in different Caucasian populations. Despite the findings of the previous studies, no individual variant in all four studied genes was significantly more or less frequent in Czech EARR patients than in controls. Nevertheless, our results regarding polymorphism *TNFRSF11B* -163C/T (rs3102735) are in agreement with study by Pereira *et al* (2014). Similarly to conclusion of haplotype analysis performed by Iglesias-Linares *et al* (2014), no differences in haplotype frequencies composed of two SNPs in the *SPP1* gene (rs11730582 and rs9138) in Czech population were detected.

While no individual gene variant was found to provide a significant influence on the EARR in our population, one haplotype of *P2RX7* gene modified the risk of orthodontically induced EARR development. The P2RX7 protein is not only an important regulator of bone remodeling but it is also able to stimulate inflammatory response release of pro-inflammatory cytokines, such as IL-1β (Ferrari *et al*, 2006). Viecilli *et al* (2009) used a P2RX7 knockout mice model for the demonstration of its influence on orthodontic mechanotransduction and confirmed *P2RX7* as a candidate gene for orthodontically induced EARR.

To this date, two recent studies have associated SNPs in the *P2RX7* gene with postorthodontic EARR. Pereira *et al* (2014) found that clinical and genetic parameters, among others the GG variant (rs1718119) in the *P2RX7*

Table 3 Details of detection for SNPs in the *IL-17A*, *P2RX7*, *SPP1*, and *TNFRSF11B* genes

SNP	*TaqMan® Genotyping Assay ID*	*Context sequence (SNP position)*	*Localization*
IL-17A -197G/A (rs2275913)	C_15879983_10	TGCCCTTCCCATTTTCCTTCAGAAG[A/G] AGAGATTCTTCTATGACCTCATTGG	Promotor
P2RX7 +489C/T (rs208294, Tyr155His)	C_3019032_1	AATTCAGACCGGAAGGTGTGTAGTG[C/T] ATGAAGGGAACCAGAAGACCTGTGA	Exon 5
P2RX7 +1068G/A (rs1718119, Thr348Ala)	C_11704039_10	CGCTTGTCTGCATTCTCCCCAGGCC[A/G] CTGTGTTCATCGACTTCCTCATCGA	Exon 11
SPP1 -443T/C (rs11730582)	C_1840808_10	GAGTAGTAAAGGACAGAGGCAAGTT[C/T] TCTGAACTCCTTGCAGGCTTGAACA	Promotor
SPP1 +1239A/C (rs9138)	C_8826997_10	TCTCATGAATAGAAATTTATGTAGA[A/C] GCAAACAAAATACTTTTACCCACTT	3′UTR region
TNFRSF11B -163T/C (rs3102735)	C_1971046_10	CTCTAGGGTTCGCTGTCTCCCCCAT[C/T] AATTCCCTGGTCTAGAAGTTAGACT	Promotor
TNFRSF11B +1181C/G (rs2073618, Lys3Asn)	C_1971047_1	TTACCACGAGCGCGCAGCACAGCAA[C/G] TTGTTCATTGTGGTCCCCGGAAACC	Exon 1

EARR, external apical root resorption; IL-17, interleukin-17; P2RX7, purinoreceptor P2X7; SNP, single nucleotide polymorphism; SPP1, secreted phosphoprotein 1 or osteopontin; TNFRSF11B, tumor necrosis factor receptor superfamily member 11B or osteoprotegerin.

Table 4 Genotype and allele frequencies of *IL-17A*, *P2RX7*, *SPP1*, and *TNFRSF11B* polymorphisms in control and EARR groups

SNP	*Genotype Allele*	*Controls* N = *69 (%)*	*EARR patients* N = *30 (%)*
IL-17A	GG	28 (40.6)	12 (40.0)
-197G/A	AG	28 (40.6)	15 (50.0)
(rs2275913)	AA	13 (18.8)	3 (10.0)
	G	84 (60.9)	39 (65.0)
	A	54 (39.1)	21 (35.0)
P2RX7	CC	25 (36.2)	13 (43.3)
+489C/T	CT	34 (49.3)	11 (36.7)
(rs208294, Tyr155His)	TT	10 (14.5)	6 (20.0)
	C	84 (60.9)	37 (61.7)
	T	54 (39.1)	23 (38.3)
P2RX7	GG	20 (29.0)	6 (20.0)
+1068G/A	AG	37 (53.6)	18 (60.0)
(rs1718119, Thr348Ala)	AA	12 (17.4)	6 (20.0)
	G	77 (55.8)	30 (50.0)
	A	61 (44.2)	30 (50.0)
SPP1	TT	22 (31.9)	8 (26.7)
-443T/C	TC	38 (55.1)	17 (56.7)
(rs11730582)	CC	9 (13.0)	5 (16.6)
	T	82 (59.4)	33 (55.0)
	C	56 (40.6)	27 (45.0)
SPP1	AA	36 (52.9)	14 (50.0)
+1239A/C	AC	28 (41.2)	14 (50.0)
(rs9138)[a]	CC	4 (5.9)	0 (0.0)
	A	100 (73.5)	42 (75.0)
	C	36 (26.5)	14 (25.0)
TNFRSF11B	TT	50 (72.5)	20 (66.7)
-163T/C	CT	16 (23.2)	8 (26.7)
(rs3102735)	CC	3 (4.3)	2 (6.6)
	T	116 (84.1)	48 (80.0)
	C	22 (15.9)	12 (20.0)
TNFRSF11B	CC	14 (20.3)	5 (16.7)
+1181C/G	CG	34 (49.3)	15 (50.0)
(rs2073618, Lys3Asn)	GG	21 (30.4)	10 (33.3)
	C	62 (44.9)	25 (41.7)
	G	76 (55.1)	35 (58.3)

EARR, external apical root resorption; IL-17, interleukin-17; *N*, number of subjects; P2RX7, purinoreceptor P2X7; SNP, single nucleotide polymorphism; SPP1, secreted phosphoprotein 1 or osteopontin; TNFRSF11B, tumor necrosis factor receptor superfamily member 11B or osteoprotegerin.
Comparisons performed by Fisher's exact test.
[a]Genotype is known only in 68 controls and 28 patients with EARR.

Table 5 Haplotype frequencies of *P2RX7* gene variants in controls and EARR patients

Haplotype	*P2RX7* *(rs208294)*	*P2RX7* *(rs1718119)*	*Controls* N = *69* N (%)	*EARR patients* N = *30* N (%)
1[a]	C	G	(31.3)	(37.7)
2	C	A	(29.6)	(26.0)
3	T	G	(24.5)	(24.0)
4	T	A	(14.6)	(12.3)

EARR, external apical root resorption; *N*, number of subjects; P2RX7, purinoreceptor P2X7.
[a]*P*-value < 0.05 in recessive models (without correction for multiple comparisons).

gene, affected the EARR development. Sharab and coworkers analyzed even three SNPs in the *P2RX7* gene (rs208294, rs1718119, rs2230912), but with conflicting results. The allele and genotype frequencies of two SNPs changing amino acid sequence in the P2RX7 protein (+1068A/G, rs1718119, Thr348Ala, and +1405A/G, rs2230912, Gln460Arg) were similar between healthy controls and EARR patients from the United States, but the other polymorphism (+489C/T, rs208294, Tyr155His) was associated with EARR development (Sharab *et al*, 2015).

Results of our complex analysis in the *P2RX7* gene cannot be compared with distribution of haplotypes in other populations, because no other study analyzing them in relation to EARR exists. It is assumed that haplotype determination can be more advantageous than an analysis based on individual polymorphisms in the presence of multiple susceptibility alleles, especially when the linkage of variants is low (Morris and Kaplan, 2002), as is the case of the studied polymorphisms in the *P2RX7* gene ($D' = 0.03$ in the whole set). Consistent with conclusions of both above-mentioned studies (Pereira *et al*, 2014; Sharab *et al*, 2015), the CG haplotype of *P2RX7* gene was more often found in Czech patients with EARR than in controls. This haplotype (containing SNP rs1718119) with G allele, which encodes alanine in the protein position +348, predisposing to gain of function and increased pro-inflammatory IL-1β secretion (Stokes *et al*, 2010; Sun *et al*, 2010; Diercke *et al*, 2012), may be one of the risk factors for the development of EARR.

The association of interindividual variability in gene encoding P2RX7 with postorthodontic EARR in Czech children should be carefully taken due to the small size of studied population, even though the number of enrolled patients is comparable with studies in other populations (Table 1). The next drawback of this study is that the levels of studied proteins were not measured; on the other side, the functional significance of gene variants selected for analysis was previously described and is mostly known. A further limitation of the study is a method employed for determining EARR diagnosis using two-dimensional orthopantomograms and lateral cephalograms; nevertheless, the testing methods provided sufficient results similar to findings on three-dimensional images obtained from a cone-beam computed tomography (CBCT) (Cattaneo *et al*, 2008; Park *et al*, 2012).

In conclusion, although the effect of *IL-17*, *P2RX7*, *SPP1*, or *TNFRSF11B* SNPs themselves on the development of EARR was not confirmed in the Czech population, complex analysis suggests that the variability in the *P2RX7* gene and the length of treatment may be factors contributing to the etiopathogenesis of postorthodontic EARR. However, for the multifactorial diseases such as EARR, it is typical that each genetic polymorphism has generally only a small effect and interactions of gene variants with environmental factors affect the observed clinical phenotype. Due to a small number of subjects included in this study, number of comparisons performed, and only marginally significant differences, our results should be interpreted carefully and need to be proven in a larger independent cohort.

Acknowledgements

The study was supported by funds from the Faculty of Medicine MU to junior researcher Petra Borilova Linhartova, project MUNI/A/1258/2015 and grant GACR GB14-37368G.

Author contributions

P.B.L., P.C., and L.I.H. designed the study and drafted the paper. P.C. performed the clinical analysis and collected the blood samples. P.B.L., J.K., Z.V., and M.S. carried out the molecular analysis, and L.I.H. performed statistical analysis. All authors revised the final version of the manuscript. Authors also confirm to have no conflict of interest.

References

Abass SK, Hartsfield JK Jr, Al-Qawasmi RA, Everett ET, Foroud TM, Roberts WE (2008). Inheritance of susceptibility to root resorption associated with orthodontic force in mice. *Am J Orthod Dentofacial Orthop* **134**: 742–750.

Al-Qawasmi RA, Hartsfield JK Jr, Everett ET *et al* (2003a). Genetic predisposition to external apical root resorption. *Am J Orthod Dentofacial Orthop* **123**: 242–252.

Al-Qawasmi RA, Hartsfield JK Jr, Everett ET *et al* (2003b). Genetic predisposition to external apical root resorption in orthodontic patients: linkage of chromosome 18 marker. *J Dent Res* **82**: 356–360.

Bastos Lages EM, Drummond AF, Pretti H *et al* (2009). Association of functional gene polymorphism IL-1beta in patients with external apical root resorption. *Am J Orthod Dentofacial Orthop* **136**: 542–546.

Bastos JV, Côrtes MI, Silva JF *et al* (2015). A study of the interleukin-1 gene cluster polymorphisms and inflammatory external root resorption in replanted permanent teeth. *Int Endod J* **48**: 878–887.

Borilova Linhartova P, Kastovsky J, Lucanova S *et al* (2016). IL-17A gene variability in patients with type 1 diabetes mellitus and chronic periodontitis; its correlation with IL-17 levels and the occurrence of periodontopathic bacteria. *Mediators Inflamm* doi: 10.1155/2016/2979846.

Brezniak N, Wasserstein A (2002). Orthodontically induced inflammatory root resorption. Part I: the basic science aspects. *Angle Orthod* **72**: 175–179.

Cattaneo PM, Bloch CB, Calmar D, Hjortshøj M, Melsen B (2008). Comparison between conventional and cone-beam computed tomography-generated cephalograms. *Am J Orthod Dentofacial Orthop* **134**: 798–802.

Diercke K, Kohl A, Lux CJ, Erber R (2012). IL-1β and compressive forces lead to a significant induction of RANKL-expression in primary human cementoblasts. *J Orofac Orthop* **73**: 397–412.

Ferrari D, Pizzirani C, Adinolfi E *et al* (2006). The P2X7 receptor: a key player in IL-1 processing and release. *J Immunol* **176**: 3877–3883.

Fontana ML, de Souza CM, Bernardino JF *et al* (2012). Association analysis of clinical aspects and vitamin D receptor gene polymorphism with external apical root resorption in orthodontic patients. *Am J Orthod Dentofacial Orthop* **142**: 339–347.

Grol MW, Panupinthu N, Korcok J, Sims SM, Dixon SJ (2009). Expression, signaling, and function of P2X7 receptors in bone. *Purinergic Signal* **5**: 205–221.

Gülden N, Eggermann T, Zerres K, Beer M, Meinelt A, Diedrich P (2009). Interleukin-1 polymorphisms in relation to external apical root resorption (EARR). *J Orofac Orthop* **70**: 20–38.

Harris EF, Robinson QC, Woods MA (1993). An analysis of causes of apical root resorption in patients not treated orthodontically. *Quintessence Int* **24**: 417–428.

Harris EF, Kineret SE, Tolley EA (1997). A heritable component for external apical root resorption in patients treated orthodontically. *Am J Orthod Dentofacial Orthop* **111**: 301–309.

Hartsfield JK Jr (2009). Pathways in external apical root resorption associated with orthodontia. *Orthod Craniofac Res* **12**: 236–242.

Hartsfield JK Jr, Everett ET, Al-Qawasmi RA (2004). Genetic factors in external apical root resorption and orthodontic treatment. *Crit Rev Oral Biol Med* **15**: 115–122.

Hayashi N, Yamaguchi M, Nakajima R, Utsunomiya T, Yamamoto H, Kasai K (2012). T-helper 17 cells mediate the osteo/odontoclastogenesis induced by excessive orthodontic forces. *Oral Dis* **18**: 375–388.

Iglesias-Linares A, Yañez-Vico R, Ballesta-Mudarra S *et al* (2012). Postorthodontic external root resorption is associated with IL1 receptor antagonist gene variations. *Oral Dis* **18**: 198–205.

Iglesias-Linares A, Yañez-Vico RM, Ballesta-Mudarra S *et al* (2013). Interleukin 1 receptor antagonist (IL1RN) genetic variations condition post-orthodontic external root resorption in endodontically-treated teeth. *Histol Histopathol* **28**: 767–773.

Iglesias-Linares A, Yanez-Vico RM, Moreno-Fernandez AM, Mendoza-Mendoza A, Orce-Romero A, Solano-Reina E (2014). Osteopontin gene SNPs (rs9138, rs11730582) mediate susceptibility to external root resorption in orthodontic patients. *Oral Dis* **20**: 307–312.

Katagiri T, Takahashi N (2002). Regulatory mechanisms of osteoblast and osteoclast differentiation. *Oral Dis* **8**: 147–159.

Kocadereli I, Yesil TN, Veske PS, Uysal S (2011). Apical root resorption: a prospective radiographic study of maxillary incisors. *Eur J Dent* **5**: 318–323.

Kvist TM, Schwarz P, Jørgensen NR (2014). The P2X7 receptor: a key player in immune-mediated bone loss? *Sci World J* **2014**: 954530.

Linhartova P, Cernochova P, Izakovicova Holla L (2013). IL1 gene polymorphisms in relation to external apical root resorption concurrent with orthodontia. *Oral Dis* **19**: 262–270.

Marek I, Špidlen M, Kamínek M (2001). Resorpce apexu kořene řezáků. 3. Vliv typů léčby a rizikové faktory. *Ortodoncie* **11**: 26–33.

Maués CP, do Nascimento RR, Vilella Ode V (2015). Severe root resorption resulting from orthodontic treatment: prevalence and risk factors. *Dental Press J Orthod* **20**: 52–58.

Morris RW, Kaplan NL (2002). On the advantage of haplotype analysis in the presence of multiple disease susceptibility alleles. *Genet Epidemiol* **23**: 221–233.

Nakano Y, Yamaguchi M, Shimizu M *et al* (2015). Interleukin-17 is involved in orthodontically induced inflammatory root resorption in dental pulp cells. *Am J Orthod Dentofacial Orthop* **148**: 302–309.

Newman WG (1975). Possible etiologic factors in external root resorption. *Am J Orthod* **67**: 522–539.

Ngan DC, Kharbanda OP, Byloff FK, Darendeliler MA (2004). The genetic contribution to orthodontic root resorption: a retrospective twin study. *Aust Orthod J* **20**: 1–9.

Park CS, Park JK, Kim H, Han SS, Jeong HG, Park H (2012). Comparison of conventional lateral cephalograms with corresponding CBCT radiographs. *Imaging Sci Dent* **42**: 201–205.

Pereira S, Lavado N, Nogueira L, Lopez M, Abreu J, Silva H (2014). Polymorphisms of genes encoding P2X7R, IL1B, OPG a RANK in orthodontic-induced apical root resorption. *Oral Dis* **20**: 659–667.

Picanço GV, de Freitas KM, Cançado RH, Valarelli FP, Picanço PR, Feijão CP (2013). Predisposing factors to severe external root resorption associated to orthodontic treatment. *Dental Press J Orthod* **18**: 110–120.

Quinn JM, Saleh H (2009). Modulation of osteoclast function in bone by the immune system. *Mol Cell Endocrinol* **310**: 40–51.

Segal GR, Schiffman PH, Tuncay OC (2004). Meta analysis of the treatment-related factors of external apical root resorption. *Orthod Craniofac Res* **7**: 71–78.

Sharab LY, Morford LA, Dempsey J *et al* (2015). Genetic and treatment-related risk factors associated with external apical root resorption (EARR) concurrent with orthodontia. *Orthod Craniofac Res* **18**(Suppl 1): 71–82.

Smale I, Artun J, Behbehani F, Doppel D, van't Hof M, Kuijpers-Jagtman AM (2005). Apical root resorption 6 months after initiation of fixed orthodontic appliance therapy. *Am J Orthod Dentofacial Orthop* **128**: 57–67.

Stokes L, Fuller SJ, Sluyter R, Skarratt KK, Gu BJ, Wiley JS (2010). Two haplotypes of the P2X(7) receptor containing the Ala-348 to Thr polymorphism exhibit a gain-of-function effect and enhanced interleukin-1beta secretion. *FASEB J* **24**: 2916–2927.

Sun C, Chu J, Singh S, Salter RD (2010). Identification and characterization of a novel variant of the human P2X(7) receptor resulting in gain of function. *Purinergic Signal* **6**: 31–45.

Tomoyasu Y, Yamaguchi T, Tajima A, Inoue I, Maki K (2009). External apical root resorption and the interleukin-1B gene polymorphism in the Japanese population. *Orthodontic Waves* **68**: 152–157.

Tyrovola JB, Spyropoulos MN, Makou M, Perrea D (2008). Root resorption and the OPG/RANKL/RANK system: a mini review. *J Oral Sci* **50**: 367–376.

Viecilli RF, Katona TR, Chen J, Hartsfield JK Jr, Roberts WE (2009). Orthodontic mechanotransduction and the role of the P2X7 receptor. *Am J Orthod Dentofacial Orthop* **135**: 694.e1-16; discussion 694-695.

Wu FL, Wang LY, Huang YQ, Guo WB, Liu CD, Li SG (2013). Interleukin-1β +3954 polymorphisms and risk of external apical root resorption in orthodontic treatment: a meta-analysis. *Genet Mol Res* **12**: 4678–4686.

3. Dental caries

Note: This chapter builds on my Dissertation thesis (Bořilová Linhartová, 2014).

Dental caries is a multifactorial disease, which despite a series of preventive measures remains the most widespread infectious disease in the world. Increasing caries experience in the pediatric population is a result of the coincidence of risk cariogenic factors and improper lifestyle associated with social action, educational, behavioural and economic factors.

Dental caries was associated with dysbiosis. Several oral species are acidogenic and aciduric (*Streptococcus mutans*, *Lactobacillus* sp. and *Actinomycetes* sp.) and appear to work synergistically to promote tooth demineralization. Costalonga and Herzberg (2014) suggest that emergent consortia of minor members of the respective microbiomes act synergistically to stress the ability of the host to respond and protect.

Innate immunity rapidly defends the host against infectious insults. These reactions are of limited specificity and exhaust without providing long-term protection. Functional fluids and effector molecules contribute to the defence against infectious agents, drive the immune response, and direct the cellular players. Secretory IgA antibody and other salivary antimicrobial systems act against cariogenic bacteria (Meyle et al., 2017).

Causal factor for the formation and development of dental caries are metabolites (organic acids) of carbohydrates produced by cariogenic microorganisms of dental plaque. Acidic environment cause lesions of tooth enamel and destruction of dentin depending on the length of exposure.

In addition, the risks associated with the use of some drugs and dosage forms in relation to the formation and development of dental caries is highlighted in our review. Drugs were classified by the mechanism of their action in this process, into those that 1. influence the tooth development, especially enamel, 2. directly or indirectly damage the tooth structure and/or 3. disrupt the protective function of saliva, causing oral microflora dysbiosis (Bořilová Linhartová and Izakovičová Hollá, 2017, Attachment 4).

Although, there is a declining trend in the incidence of dental caries in children, Czech Republic undoubtedly has the potential for further improvement of the dentition in implementation of adequate preventive measures against dental caries (Lenčová and Broukal, 2012; Bořilová Linhartová and Bartošová, 2019). One of the goals of the Health 21 program (2002) is to at least 80 % of children aged 6 years were caries free and children at age 12 had by an average maximally 1.5 DMFT (decay, missing or filled teeth).

Early childhood caries (ECC) is defined by American Academy of Pediatric Dentistry (AAPD) as the presence of one or more decayed (non-cavitated or cavitated lesions [d_1-d_4]), missing (due to caries), or filled tooth surfaces in any primary tooth in a child under the age of six. Severe ECC (sECC) is any sign of smooth-surface caries in a child younger than 3 years of age (AAPD, 2017).

By the evaluation of possible associated factors for ECC dealt study by Özen et al. (2016), who associated lack of periodic dental examination, sugar and fruit-juice consumption and prolonged (>18 months) breastfeeding with caries formation. Divaris (2016) suggested that accurate caries risk assessment at the population-level and "precision dentistry" at the person-level are both desirable and achievable but must be based on high-quality longitudinal data and rigorous methodology.

In the 275 Czech children with sECC, the upper middle teeth and the first molars in the upper and lower jaws were most commonly affected by dental caries. On the other hand, less than 20% of sECC children had carious lesions on incisors or canines in the lower jaw (Fig. 9).

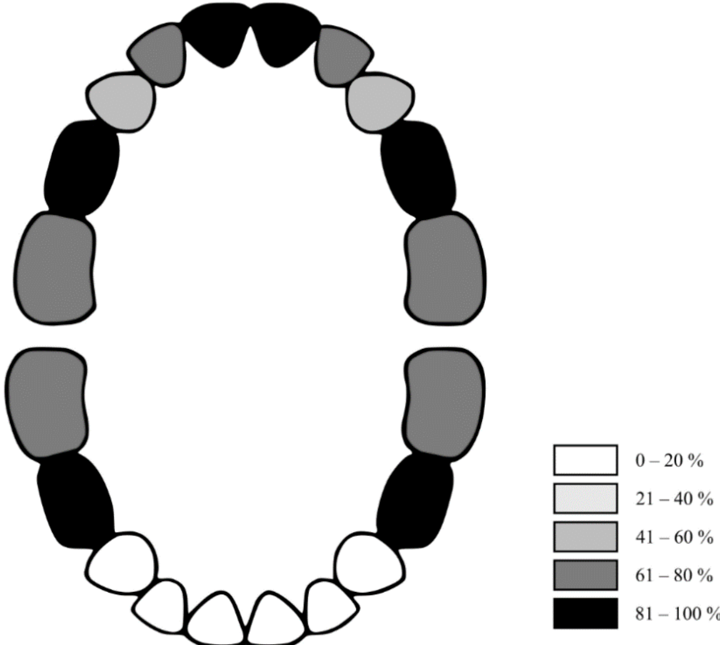
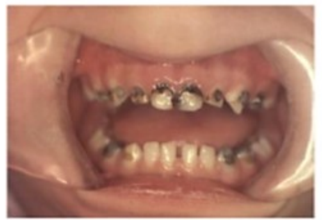
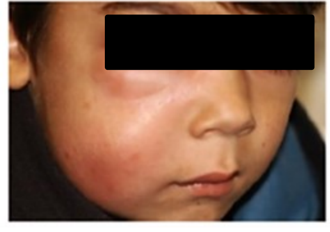


Fig. 9. Graphical representation of the incidence of dental caries in primary dentition in children with sECC. The scale defines % of children, who have dental caries given to the tooth (Bořilová Linhartová et al., 2018).

The dominant affection of upper incisors with tooth decay has been described in the past by the term "baby bottle teeth". On the other hand, the teeth in the frontal area of the lower jaw, which are most in contact with the saliva and are protected from dental caries.

Results of our study suggest that breastfeeding of children in the duration of 6 to 24 months appears to be a protective factor for this infectious disease. On the contrary, serving sweetened drinks and late start of oral hygiene was associated with sECC. Breastfeeding longer than two years was connected to risk behaviour of mothers/caregivers which can result in the development of sECC (Bořilová Linhartová et al., 2018, Attachment 5).
Appropriately targeted behavioural intervention using effective tools can help to eliminate risk behaviour leading to ECC. The aim of our other study was to ascertain which visual stimuli with a supporting text evoke the strongest emotional response in infants' mothers and, therefore, are suitable candidates for inclusion in behavioural interventions within the prevention of ECC. This pilot study proved that negative pictorial and text warnings about the risks of developing caries had the potential to evoke strong emotional responses in the mothers of infants. We identified three stimuli (Fig. 10) that could be included in future extensive motivation material in an attempt to affect the preventive behaviour of mothers, and thus the oral health of their infants (Bartosova et al., 2019, Attachment 6).





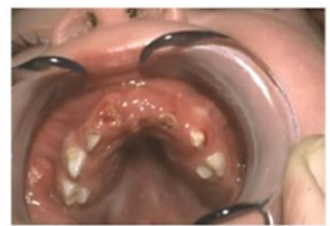


Fig. 10. Stimuli that could be included in future extensive motivation material in an attempt to affect the preventive behaviour of infant's mothers (Bartosova et al., 2019).

In the context of strengthening prevention and oral care, several commercial tests for the determination of the individual susceptibility to these diseases can be an effective tool in behavioral intervention not only in patients with risk finding. The advantages and

disadvantages of products currently available to dentists on the Czech (or EU) market for testing in relation to dental caries and/or periodontal disease in children and adults were described in our recent review (Bořilová Linhartová et al., 2019, Attachment 7). In addition, preventive program „Dragons teeth“ for preschool children was created and this student's project has been running for two years under my leadership.

Note: This project and follow-up study was presented by my student (Mona Aliwiová) on the Student scientific conference 2018 in Brno and was awarded 3rd place.

Host susceptibility is influenced by external (composition and frequency of food intake, socio-economic, socio-cultural and behavioral factors) and internal modifying factors (composition and quantity of saliva, chronic systemic diseases, immune status, morphology and anatomy of dental hard tissues, genetic predisposition, Fig. 11).

Regarding the importance of genetic predisposition to dental caries (share of inheritance 20-65 %), Wang et al. (2010) suggested in their study that the genes affecting susceptibility to caries in the primary dentition may differ from those in the permanent teeth. Identification of genes and genetic markers with diagnostic, prognostic and therapeutic significance have been estabilished as one of the six main directions that current caries research should take. Over the last decade we have seen an increasing scientific interest in the study of genetic factors influencing the susceptibility to dental caries and protection against this disease. The major candidate gene categories studied to date include enamel formation genes, immune response genes, genes related to saliva, genes related to taste, and others (Vieira et al., 2014; Chaplle et al., 2017).

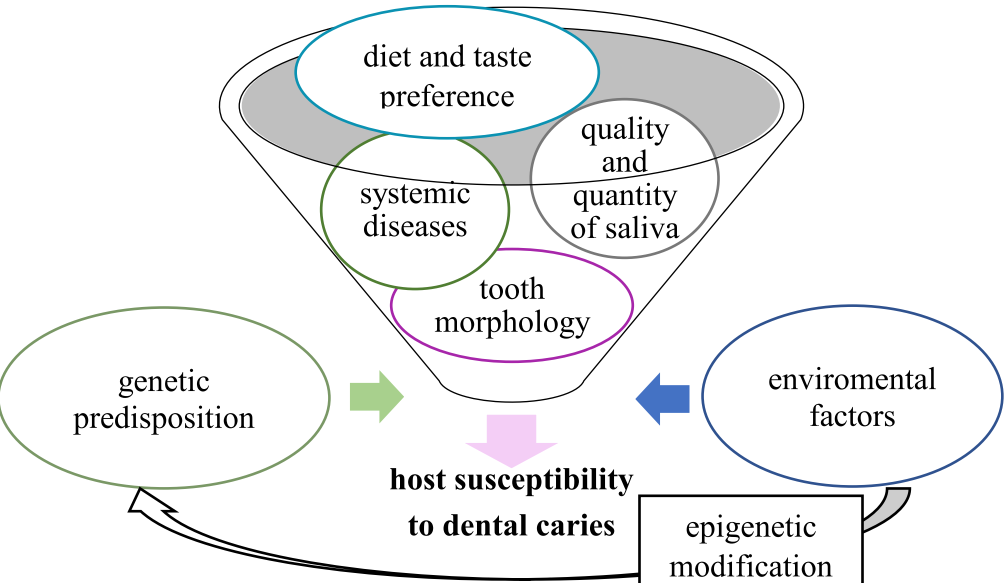


Fig. 11. Factors involved in the host susceptibility to the development of dental caries (Borilova Linhartova and Bartosova, 2019).

The first GWAS looking for genes associated with the risk of caries development was published in the year 2011. This study indicated that there are a number of loci that mostly have little effect and are involved in cariogenensis (Shaffer et al., 2011). In the other GWAS was an association between dental caries and a region in the visfatin gene found (Morrison et al., 2016), which is also considered as a marker for obesity (Li et al., 2013). Currently published GWAS included 19,003 children with a primary teeth and 13,353 patients with a permanent teeth, and several gene variants were associated with the risk of dental caries (Haworth et al., 2018). Specifically, variability in the gene encoding allantoicase enzyme (ALLC) involved in purine metabolism, whose enzymatic activity was probably lost during vertebrate evolution, was associated with dental caries in temporary dentition. For dental caries in permanent dentition, the *NEDD9* candidate gene encodes a neural precursor cell expressed expressionally down-regulated protein 9, which plays a role in regulating neural differentiation, neural cell development and migration (Haworth et al., 2018).

In addition to congenital predispositions, the importance of epigenetics in relation to dental caries is also studied. Epigenetic mechanisms, such as methylation of DNA, histone modification, and the presence of RNA are not coded, gene expression and potentially pathogenesis of the disease are affected (Seo et al., 2015). Hypothetically, epigenetic changes leading to the development of the oral cavity – especially the development of the teeth and salivary glands – can also be associated with dental caries, immune functions in the body can also be altered, thus impairing its natural defenses. Fernando and colleagues (2015) proposed a protocol to evaluate a range of variables, including epigenetic factors, in relation to the risk of developing and developing dental caries in children.

Low validity for the analysed methods may lead to patients with increased risk not being identified, whereas some are falsely identified as being at risk. As caries risk assessment guides individualized decisions on interventions and intervals for patient recall, improved performance based on best evidence is greatly needed (Senneby et al., 2015). Recent advances in deep-sequencing technologies, novel imaging methods and (meta)proteomics-metabolomics approaches could have direct implications for developing new approaches for improved risk assessment and prevention of this devastating and costly childhood health condition (Hajishengallis et al., 2015).

The clinical relevance of genetic information is still problematic; the potential for incorporating this factor into the new risk assessment prognosis program will increase with the increase in knowledge, especially when the complex genetic profile is known (Kornman

and Polverini, 2014). Analysis of genetic risk factors could help in the future in screening and identifying patients with disposition to develop dental caries.

In the Czech population of children with primary and/or permanent teeth, we have so far observed variability in genes involved in amelogenesis (*DLX3*, bone morphogenetic protein 2, enamelin) (Kastovsky et al., 2017, Attachment 8; Borilova Linhartova et al., 2018a, Attachment 9). As a result of most of our observations, the association of specific polymorphisms with disease previously found in other populations was not confirmed.
Note: The study focused on enamelin gene variant and dental caries in children was presented by my student (Tereza Deissová) on the Student scientific conference 2017 in Brno and was awarded 2nd place.

On the other hand, variants in sweet taste receptor (*TAS1R2*) and glucose transporter (*GLUT2*) were associated with increased risk of dental caries in permanent dentition in the population of Czech children from European Longitudinal Study of Pregnancy and Childhood (ELSPAC, Izakovicova Holla et al., 2015, Attachment 10).
From genes encoded immunoregulatory proteins, only lactotransferrin (*LTF*) was previously studied in Czech children from ELSPAC and lack of association between SNP *LTF* rs1126478 and dental caries in permanent dentition was found (Volckova et al., 2014).
In the recent review from Lips et al. (2017), association between genetic polymorphisms and risk of dental caries for most of the salivary proteins was demonstrated. Association was found for proteins related to antimicrobial activity (beta defensin 1 and lysozyme-like protein), pH control (carbonic anhydrase VI), and bacterial colonization/adhesion (LTF, mucin, and proline-rich protein Db).
In addtion, our research of dental caries is focused on changes in *S. mutans* transcriptome and metabolome depending on carbohydrate substrate and on a role of *Candida* sp. and cariogenic bacteria in sECC development.

3.1 Comment to the article "*ACE* insertion/deletion polymorphism associated with caries in permanent but not primary dentition in Czech children"

Borilova Linhartova P, Kastovsky J, Bartosova M, Musilova K, Zackova L, Kukletova M, Kukla L, Izakovicova Holla L. *ACE* insertion/deletion polymorphism associated with caries in permanent but not primary dentition in Czech children. Caries Res. 2016;50(2):89-96. (IF 1.811; DENTISTRY, ORAL SURGERY & MEDICINE Q2)

Author contribution: P.B.L. designed the study, drafted the paper, and particularly carried out genetic analysis.

The angiotensin-converting enzyme (ACE) is a zinc metallopeptidase and a regulatory component of the renin-angiotensin (RA) system, where it acts by hydrolyzing angiotensin I (Ang I) to angiotensin II (Ang II) and inactivating the bradykinin (Ceconi et al., 2007). Ang II not only increases blood pressure but is also a potent proinflammatory modulator which, through activation of nicotinamide adenine dinucleotide phosphate oxidase and production of reactive oxygen species (ROS), can induce tissue damage. Besides systemic RAS, the local RAS contributes to the inflammatory process via stimulation of the production of cytokines (Mendoza-Pinto et al., 2010).

Insertion/deletion (I/D) polymorphism of the *ACE* gene, identified as a 287 base pair Alu repeat in intron 16 (17q23) (Rigat et al., 1990), is probably in a strong linkage disequilibrium with another *ACE* gene variant affecting ACE protein levels (Tiret et al., 1992). *ACE* I/D polymorphism has been associated with caries in Polish children (Olszowski et al., 2015).

The aim of this study was to analyze *ACE* I/D polymorphism in a group of caries-free children versus subjects affected by dental caries in primary/permanent dentition in the Czech population.

In this case-control study, 220 children aged 2–6 years with ECC (dmft≥1), and 743 children from ELSPAC aged 13–15 years: 561 subjects with dental caries (DMFT≥1), and 182 caries-free children (DMFT=0), were included. The genotype determination of *ACE* I/D polymorphism in intron 16 was based on the TaqMan method (Koch et al., 2015).

Although no significant differences in the allele or genotype frequencies between the caries-free children and those affected by dental caries were observed, statistically significant differences between the the subgroup of 179 patients with high caries experience (DMFT≥4) and caries-free children (P<0.01 and P<0.05, respectively) were detected. The comparison of DD versus II+ID genotype frequencies between the patients with DMFT≥1 or DMFT≥4 and caries-free children also showed significant differences (31.5% or 35.6% vs. 23.6%,

$P<0.05$ or $P<0.01$, respectively). A gender-based analysis identified a significant difference in the DD versus II+ID genotype frequencies only in girls ($P<0.05$). In contrast, no significant association of *ACE* I/D polymorphism with ECC in young children was found ($P>0.05$).

Our findings are in disagreement with those published by Olszowski et al. (2015), which could be a result of the insufficient sample size and problematic genotyping method in the Polish study.

Our study suggested that the *ACE* I/D polymorphism may be associated with caries in permanent but not primary dentition, especially in girls in the Czech population. Nevertheless, the mechanism of a possible ACE effect on susceptibility to dental caries is unknown, and further research of people with different ethnic backgrounds is required.

Original Paper

Caries Research

Caries Res 2016;50:89–96
DOI: 10.1159/000443534

Received: September 18, 2015
Accepted: December 21, 2015
Published online: February 27, 2016

ACE Insertion/Deletion Polymorphism Associated with Caries in Permanent but Not Primary Dentition in Czech Children

Petra Borilova Linhartova[a, b] Jakub Kastovsky[a, b] Michaela Bartosova[a, d] Kristina Musilova[a] Lenka Zackova[c] Martina Kukletova[a] Lubomir Kukla[d] Lydie Izakovicova Holla[a, b]

[a]Clinic of Stomatology, Institutions Shared with St. Anne's Faculty Hospital, [b]Department of Pathophysiology, and [c]Stomatology Research Center, Faculty of Medicine, and [d]Research Centre for Toxic Compounds in the Environment (RECETOX), Chemistry Section, Faculty of Science, Masaryk University, Brno, Czech Republic

Key Words

Angiotensin-converting enzyme · Children · Dental caries · ELSPAC · Gene polymorphism

Abstract

Objective: Dental caries is a multifactorial, infectious disease where genetic predisposition plays an important role. Insertion/deletion (I/D) polymorphism of angiotensin-converting enzyme (ACE) has very recently been associated with caries in Polish children. The aim of this study was to analyze *ACE* I/D polymorphism in a group of caries-free children versus subjects affected by dental caries in the Czech population. ***Materials and Methods:*** In this case-control study, 182 caries-free children (with decayed/missing/filled teeth, DMFT = 0), 561 subjects with dental caries (DMFT ≥1) aged 13–15 years and 220 children aged 2–6 years with early childhood caries (ECC, dmft ≥1) were included. Genotype determination of *ACE* I/D polymorphism in intron 16 was based on the TaqMan method. ***Results:*** Although no significant differences in the allele or genotype frequencies between the caries-free children and those affected by dental caries were observed, statistically significant differences between the children with DMFT = 0 and the subgroup of 179 patients with high caries experience (DMFT ≥4; $p < 0.01$ and $p < 0.05$, respectively) were detected. The comparison of DD versus II+ID genotype frequencies between the patients with DMFT ≥1 or DMFT ≥4 and healthy children also showed significant differences (31.5% or 35.6% vs. 23.6%, $p < 0.05$ or $p < 0.01$, respectively). A gender-based analysis identified a significant difference in the DD versus II+ID genotype frequencies only in girls ($p < 0.05$). In contrast, no significant association of *ACE* I/D polymorphism with ECC in young children was found ($p > 0.05$). ***Conclusions:*** *ACE* I/D polymorphism may be associated with caries in permanent but not primary dentition, especially in girls in the Czech population.

© 2016 S. Karger AG, Basel

Dental caries, the most widespread disease worldwide, is a process of demineralization affecting dental hard tissues [Ozdemir, 2013; Fejerskov et al., 2015]. It is generally accepted that cariogenic bacteria play a major role in this process. However, the etiology of dental caries is mul-

KARGER

E-Mail karger@karger.com
www.karger.com/cre

© 2016 S. Karger AG, Basel
0008–6568/16/0502–0089$39.50/0

Prof. Lydie Izakovicova Holla, MD, PhD
Clinic of Stomatology and Department of Pathophysiology
Faculty of Medicine, Masaryk University
Pekarska 53, CZ–656 91 Brno (Czech Republic)
E-Mail holla@med.muni.cz

Downloaded by:
Masaryk University
195.113.55.1 - 2/27/2016 11:45:58 PM

tifactorial with contributions from numerous components, including oral hygiene, salivary flow and composition, diet, fluoride exposure and genetic predispositions, among others.

Studies of twins reared together [Bretz et al., 2003, 2005] or of families [Wang et al., 2010] have shown that dental caries is highly heritable, with 20–65% of variation attributable to genetics. Shaffer et al. [2015] even observed significant evidence of gene-by-sex interactions for caries experience in both the primary and permanent dentitions. The number of studies investigating the presence of genetic factors influencing individual susceptibility to caries has increased markedly over the last decade. The review by Vieira et al. [2014] revisited recent caries human genetic studies (candidate genes, genome-wide linkage and association studies) and summarized candidate genes studied for caries in humans, including the immune response genes, enamel formation genes and genes related to saliva, taste and others.

Moreover, in a very recent study, angiotensin-converting enzyme (ACE) insertion/deletion (I/D) polymorphism has been associated with dental caries in Polish children [Olszowski et al., 2015]. I/D polymorphism of the *ACE* gene, identified as a 287 base pair Alu repeat in intron 16 (17q23) [Rigat et al., 1990], is probably in strong linkage disequilibrium with another *ACE* gene variant affecting ACE protein levels [Tiret et al., 1992]. Regardless of this, the highest serum ACE levels were observed in homozygotes with deletion on both alleles of the *ACE* gene in intron 16 [Rigat et al., 1990]. Although ACE is best known for the catalytic conversion of angiotensin I to angiotensin II, the use of gene-targeting techniques led to mouse models highlighting many other biochemical properties and actions of this enzyme. The review by Gonzalez-Villalobos et al. [2013] discussed the contribution of ACE to many different physiological processes, including renal development, blood pressure control, inflammation and immunity.

Because dental caries is caused by bacterial infection and ACE may influence the immune response and antibacterial protection, we hypothesized that polymorphism in the *ACE* gene might also modify a relative risk for the development of dental caries in Czech children. Therefore, the first aim of the present study was to compare the distribution of the *ACE* alleles and genotypes between a group of caries-free children (with decayed/missing/filled teeth, DMFT = 0) and children affected by dental caries (DMFT ≥1) in the Czech population selected from the European Longitudinal Study of Pregnancy and Childhood (ELSPAC). The second aim was to analyze the *ACE* I/D polymorphism in a group of children aged 2–6 years with primary dentition affected by early childhood caries (ECC, dmft ≥1).

Methods

Subjects

A total of 743 unrelated Caucasian children (396 boys and 347 girls), aged 13–15 years, were investigated, who had been selected from the ELSPAC Brno study comprising over 5,000 children and their families [ELSPAC, 1989; Kukla and Bouchalova, 1992]. The children underwent dental examination at the Clinic of Stomatology at St. Anne's University Hospital and the Faculty of Medicine, Masaryk University in Brno, as described previously [Volckova et al., 2014]. The study group comprised caries-affected subjects (with DMFT ≥1; n = 561; 297 boys and 264 girls) and caries-free children (with DMFT = 0; n = 182; 99 boys and 83 girls).

The clinical assessment was carried out by one investigator (K.M.) using the following clinical parameters: the cavitation of the lesion as the detection threshold of caries according to the criteria given in the WHO Oral Health Surveys [1997], the gingival index (GI), the plaque index (PI) and the calculus index (CSI) as described previously [Izakovicova Holla et al., 2008]. A radiographic examination was not performed as it was not part of routine dental care for these subjects and would therefore be deemed unethical. The phenotype status was assigned without knowledge of genotypes by two independent investigators (K.M. and M.K.).

In addition, a convenience sample of 220 preschool Czech children (125 boys and 95 girls), aged 2–6 years, was recruited, who had been referred to the Pediatric Section of the Clinic of Stomatology, St. Anne's Faculty Hospital and the Faculty of Medicine, Masaryk University in Brno for complex treatment under general anesthesia. All children were generally healthy but unable to undergo standard dental treatment due to their uncooperativeness and a need for multiple restorations and extractions. Oral examination was performed by two mutually calibrated experienced pediatric dentists under standard conditions in a professional dental unit (L.Z. and K.M.). The dmft index was calculated using dental caries (D_3 level) as a cutoff point for the detection of decay.

The inclusion criteria consisted of simple informed consent of the children and their parents and an expression of their willingness to participate. The study was approved by the Ethics Committee of the Faculty of Medicine, Masaryk University in Brno, and the informed consent was obtained from all parents (in the case of children) in line with the Helsinki Declaration prior to their inclusion in the study.

DNA Isolation

DNA for genetic analysis was isolated from buccal epithelial cells obtained by buccal swabs (children from the ELSPAC study) or from blood (young children with ECC). Extractions were performed according to the manufacturer's instructions for the UltraClean® BloodSpin® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, Calif., USA) with slight adaptations.

Genotyping: TaqMan Method

The *ACE* I/D polymorphism in intron 16 was genotyped using an assay based on the TaqMan technique (Thermo Fisher Scien-

Downloaded by:
Masaryk University
198.143.55.1 - 2/27/2016 11:45:58 PM

Table 1. The distribution of the *ACE* I/D genotype and allele frequencies in children with caries experience (DMFT ≥1) and a subgroup with high caries experience (DMFT ≥4) versus caries-free children (DMFT = 0)

	Children with DMFT = 0	Children with DMFT ≥1	p	OR (95% CI)	Children with DMFT ≥4	p	OR (95% CI)
Patient-based analysis	n = 182	n = 561			n = 179		
ACE I/D							
II	43 (23.6)	121 (21.6)	0.125[a]	1.00	33 (18.4)	0.039[a,*]	1.00
ID	96 (52.8)	263 (46.9)		0.97 (0.64–1.48)	82 (45.8)		1.11 (0.65–1.91)
DD	43 (23.6)	177 (31.5)		1.46 (0.90–2.37)	64 (35.8)		1.94 (1.07–3.52)
II+ID vs. DD	76.4 vs. 23.6%	68.5 vs. 31.5%	0.025[b,*]	1.49 (1.01–2.19)	64.2 vs. 35.6%	0.008[b,*]	1.80 (1.14–2.85)
Allele-based analysis	n = 364	n = 1,122			n = 358		
ACE I/D							
I allele	182 (50.0)	505 (45.0)	0.055[b]	1.00	148 (41.3)	0.012[b,*]	1.00
D allele	182 (50.0)	617 (55.0)		1.22 (0.96-1.55)	210 (58.7)		1.42 (1.06–1.90)

Values are expressed as n (%), unless otherwise indicated. * $p < 0.05$ compared to the control group with DMFT = 0.
[a] χ^2 test. [b] Fisher exact test.

tific, Waltham, Mass., USA) previously described by Koch et al. [2005]. Allele genotyping from fluorescence measurements was then obtained using the ABI PRISM 7000 Sequence Detection System. SDS version 1.2.3 software was used to analyze real-time and end-point fluorescence data. Genotyping was performed by two investigators (J.K. and P.B.L.) unaware of the phenotype. The genotype analysis was verified by using positive control subjects in each 96-well plate and rerunning ≥5% of the samples, which were 100% concordant.

Statistical Analysis

Standard descriptive statistics were applied in the analysis: absolute and relative frequencies for categorical variables and mean and standard deviation (SD) for quantitative variables. Comparisons were made between the allele and genotype frequencies in the patients and controls (case-control study) or between patients with DMFT ≥1 and children with dmft ≥1 (ECC). The allele frequencies were calculated from the observed numbers of genotypes. The differences in the allele frequencies were tested by the Fisher exact test; Hardy-Weinberg equilibrium (HWE) and genotype frequencies were calculated by the χ^2 test. The association was described by odds ratios (OR) supplemented by their 95% confidence intervals (95% CI). Only values of $p < 0.05$ were considered statistically significant. Power analysis was performed with respect to the case-control design of the study, taking the incidence rate of markers and the estimate of OR as end-point statistical measures. Statistical analyses were computed using the software packages Statistica v. 10 (StatSoft Inc., Tulsa, Okla., USA).

Results

Of the 963 individuals included in this study, 182 (18.9%) were caries-free children (with DMFT = 0), 561 (58.3%) were carries-affected subjects with permanent dentition (with DMFT ≥1; mean 3.9 ± 2.8) and 220 (22.8%) were young children with primary dentition (with dmft ≥1; mean 11.4 ± 3.5). Periodontal status was evaluated only in 743 children from the ELSPAC study. The mean GI value per child was 0.21 (SE = 0.014). Healthy gingiva (GI = 0) as the highest score was found in 36.6%, grade 1 in 43.8% and grade 2 in 19.6% of the children; grade 3 was not detected. The mean values of PI and CSI were 0.67 and 0.68, respectively. The sample size was optimized to ensure at least 80% power to detect a difference in the rate of a marker between the cohorts or to detect OR of 1.5–1.8 as statistically significant.

The observed *ACE* I/D genotype distributions in the control group and patients with dental caries experience were in HWE ($p > 0.05$). Although no significant differences were observed in the allele or genotype frequencies between 182 caries-free subjects and 561 children with DMFT ≥1, statistically significant differences were found between children with DMFT = 0 and the subgroup of 179 patients with high caries experience (DMFT ≥4; $p < 0.02$ and $p < 0.05$, respectively). Further, the comparison of the DD and II+ID genotype frequencies in patients with DMFT ≥1 or DMFT ≥4 versus healthy children also showed significant differences (31.5% or 35.6% vs. 23.6%, $p < 0.05$, OR = 1.49, 95% CI = 1.01–2.19 or $p < 0.01$, OR = 1.80, 95% CI = 1.14–2.85, respectively; table 1).

The subanalysis performed separately in the groups of girls (n = 347) and boys (n = 396) showed a significant difference in the II+ID versus DD genotype frequencies only in girls; the DD genotype was more frequent in girls with caries experience (32.6% vs. 21.7%, $p < 0.05$, OR = 1.74, 95% CI = 0.97–3.12). However, the same but nonsignificant trend was found in the subgroup of boys ($p > 0.05$; table 2).

Caries Res 2016;50:89–96
DOI: 10.1159/000443534

Downloaded by:
Masaryk University
195.113.55.1 - 2/27/2016 11:45:58 PM

Table 2. The distribution of the *ACE* I/D genotype and allele frequencies in children with caries experience (DMFT ≥1) versus caries-free children (DMFT = 0) in gender subgroups

	Girls with DMFT = 0	Girls with DMFT ≥1	p	OR (95% CI)	Boys with DMFT = 0	Boys with DMFT ≥1	p	OR (95% CI)
Patient-based analysis	n = 83	n = 264			n = 99	n = 297		
ACE I/D								
II	22 (26.5)	66 (25.0)	0.150[a]	1.00	21 (20.8)	55 (18.5)	0.571[a]	1.00
ID	43 (51.8)	112 (42.4)		0.87 (0.48–1.58)	53 (52.5)	151 (50.9)		1.09 (0.60–1.97)
DD	18 (21.7)	86 (32.6)		1.59 (0.79–3.21)	25 (24.7)	91 (30.6)		1.39 (0.71–2.72)
II+ID vs. DD	78.3 vs. 21.7%	67.4 vs. 32.6%	0.038[b,*]	1.74 (0.97–3.12)	73.3 vs. 24.7%	69.4 vs. 30.6%	0.187[b]	1.31 (0.78–2.19)
Allele-based analysis	n = 166	n = 528			n = 198	n = 594		
ACE I/D								
I allele	87 (52.4)	244 (46.2)	0.096[b]	1.00	95 (48.0)	261 (43.9)	0.182[b]	1.00
D allele	79 (47.6)	284 (53.8)		1.28 (0.90–1.82)	103 (52.0)	333 (56.1)		1.18 (0.85–1.62)

Values are expressed as n (%), unless otherwise indicated. * $p < 0.05$ compared to the control group with DMFT = 0.
[a] χ^2 test. [b] Fisher exact test.

Table 3. The distribution of the *ACE* I/D genotype and allele frequencies in children with ECC (dmft ≥1) versus caries-free children (DMFT = 0)

	Children with DMFT = 0	ECC children with dmft ≥1	p	OR (95% CI)
Patient-based analysis	n = 182	n = 220		
ACE I/D				
II	43 (23.6)	53 (24.1)	0.927[a]	1.00
ID	96 (52.8)	112 (50.9)		0.95 (0.58–1.54)
DD	43 (23.6)	55 (25.0)		1.04 (0.59–1.83)
II+ID vs. DD	76.4 vs. 23.6%	75.0 vs. 25.0%	0.420[b]	1.08 (0.68–1.70)
Allele-based analysis	n = 364	n = 440		
ACE I/D				
I allele	182 (50.0)	218 (49.5)	0.477[b]	1.00
D allele	182 (50.0)	222 (50.5)		1.02 (0.77–1.34)

Values are expressed as n (%), unless otherwise indicated. [a] χ^2 test. [b] Fisher exact test.

The distributions of genotypes and alleles of the *ACE* I/D polymorphism in children aged 2–6 years with ECC are shown in table 3. They were in HWE with nonsignificant χ^2 values compared with the observed and expected genotype frequencies. The frequencies of the *ACE* genotypes, alleles and II+ID versus DD genotypes in caries-free subjects (DMFT = 0) and children with ECC were not significantly different ($p > 0.05$). In addition, no differences were found in a sex-based analysis between subgroups of girls and boys with ECC (with dmft ≥1) when we compared them with healthy girls or boys (with DMFT = 0; table 4). However, the comparison of subjects with caries in the permanent teeth (DMFT ≥1) and children with ECC (dmft ≥1) showed significant differences in frequencies of II+ID versus DD genotypes ($p < 0.05$). In subanalysis according to gender, a significant association of the DD genotype and D allele was detected only in girls ($p < 0.05$).

Discussion

ACE is an important regulator of the renin-angiotensin-aldosterone system and the kallikrein-kininogen system by creating angiotensin II and inactivating bradykinin. It is involved in the regulation of blood pressure and

Downloaded by:
Masaryk University
195.113.55.1 - 2/27/2016 11:45:58 PM

Table 4. The distribution of the *ACE* I/D genotype and allele frequencies in children with ECC (dmft ≥1) versus caries-free children (DMFT = 0) in gender subgroups

	Girls with DMFT = 0	ECC girls with dmft ≥1	p	OR (95% CI)	Boys with DMFT = 0	ECC boys with dmft ≥1	p	OR (95% CI)
Patient-based analysis	n = 83	n = 95			n = 99	n = 125		
ACE I/D								
II	22 (26.5)	27 (28.4)	0.942[a]	1.00	21 (20.8)	26 (20.8)	0.833[a]	1.00
ID	43 (51.8)	49 (51.6)		0.93 (0.46–1.86)	53 (52.5)	63 (50.4)		0.96 (0.49–1.90)
DD	18 (21.7)	19 (20.0)		0.86 (0.37–2.02)	25 (24.7)	36 (28.8)		1.16 (0.54–2.51)
II+ID vs. DD	78.3 vs. 21.7%	80.0 vs. 20.0%	0.463[b]	0.90 (0.44–1.86)	73.3 vs. 24.7%	71.2 vs. 28.8%	0.330[b]	1.20 (0.66–2.17)
Allele-based analysis	n = 166	n = 190			n = 198	n = 250		
ACE I/D								
I allele	87 (52.4)	103 (54.2)	0.408[b]	1.00	95 (48.0)	115 (46.0)	0.374[b]	1.00
D allele	79 (47.6)	87 (45.8)		0.90 (0.44–1.86)	103 (52.0)	135 (54.0)		1.08 (0.75–1.57)

Values are expressed as n (%), unless otherwise indicated. [a] χ^2 test. [b] Fisher exact test.

inflammatory processes. The I/D polymorphism in intron 16 of the *ACE* gene accounts for most variations in serum ACE levels [Rigat et al., 1990]. Although this variant was primarily investigated in relation to cardiovascular diseases [Yang et al., 2013; Song et al., 2014; Zhao et al., 2014; Ma et al., 2015], cancer [Zhang et al., 2014] and diabetic nephropathy [Yu et al., 2012; Rahimi et al., 2014], some studies have also associated this gene polymorphism with various diseases in the oral cavity. In the Czech population, the D allele was previously found to be marginally risky for chronic periodontitis [Izakovicova Holla et al., 2001] and in the Korean population, the DD genotype has already been associated with chronic periodontitis [Kang et al., 2015]. On the other hand, in the Turkish population, the D allele was described as protective for chronic periodontitis; moreover, no association with generalized aggressive periodontitis was found [Gürkan et al., 2009a, b]. In addition, the DD genotype was reported to increase the risk of aphthous stomatitis [Karakus et al., 2013] and very recently, Olszowski et al. [2015] described a protective role of the DD genotype of the *ACE* I/D polymorphism in dental caries in Polish children.

In this study, we evaluated the I/D polymorphism of the *ACE* gene in a relatively large group of children (n = 963) with and without caries from two different data sets from the Czech population. In the initial analysis, we compared the individuals with permanent dentition aged 13–15 years from the ELSPAC study (n = 743). The subjects lived in the same area and maintained good oral hygiene. We found that although no significant differences in the allele and/or genotype frequencies between caries-free (DMFT = 0) and caries-affected (DMFT ≥1) children were detected, the number of DD homozygotes versus II+ID carriers differed significantly between both groups. Moreover, in the subgroup of Czech children with high caries experience (DMFT ≥4), the DD genotype and the D allele were identified as significant risk factors for dental caries. Further, an *ACE* gene-by-sex interaction in relation to the caries phenotype was found.

The aim of the second experiment was to assess the *ACE* I/D polymorphism in a group of preschool Czech children with primary dentition affected by ECC. In this group, I/D *ACE* alleles and genotypes were not significantly different compared with caries-free subjects. However, the comparison of the children with caries in the permanent teeth and those with ECC showed significant differences in the frequency of the DD versus II+ID genotypes, especially in girls.

The differences in the association of the *ACE* I/D polymorphism with caries according to dmft/DMFT indexes in primary/permanent dentitions in our population can be considered in relation to the statement of Wang et al. [2010], who suggested in their study that the genes affecting susceptibility to caries in the primary dentition may differ from those in the permanent teeth. Similarly, these findings were confirmed by Olszowski et al. [2012], who found that *MBL2* polymorphisms might have different effects on primary and permanent dentitions, and by the study of Romanos et al. [2015], in which the *BMP2* (rs1884302) gene polymorphism was associated with caries experience only in the primary teeth. Thus, our study reinforces the importance of stratifica-

Downloaded by:
Masaryk University
195.113.551 - 2/27/2016 11:45:58 PM

tion of caries experience by the type of dentition as primary and permanent dentitions may have different genes involved during morphogenesis and enamel formation. However, several other genetic and environmental factors (and their interaction) may be involved. These can be important, especially in our cohort of young children aged 2–6 years as they were selected from a pool of uncooperative patients with high caries experience (mean dmft = 11) and presumably poor oral hygiene, high sugar intake and neglected care. These factors appear to play a more important role than the genetic predisposition for the development of dental caries in this group. On the other hand, in children from the ELSPAC study with good oral hygiene and collaboration with dentists, the *ACE* I/D polymorphism may affect the susceptibility to dental caries.

Our results are in disagreement with those published by Olszowski et al. [2015], who found that the DD genotype versus II+ID genotypes might be protective for dental caries in Polish children aged 5–13 years. We analyzed the same *ACE* I/D polymorphism as the Polish authors, however, with some differences in sample size and methodology. Firstly, Olszowski et al. [2015] examined 161 subjects: 63 children at the age of 5 with primary dentition and 98 children at the age of 13 with permanent teeth. We analyzed the study population of 963 unrelated children, which comprised 220 children aged 2–6 years with ECC in the primary dentition and 743 children aged 13–15 years with/without caries in the permanent dentition. Secondly, analysis by gender showed the trend to a more frequent occurrence of the DD genotype in Czech girls with caries experience at the age of 13–15. This is in contrast with results of the study by Olszowski et al. [2015], who did not find the age or sex of the children to be significant predictors of dental caries susceptibility. Sex differences in dental caries experience have been previously widely reported, with females usually exhibiting a higher prevalence and severity of disease across all ages [Lukacs and Largaespada, 2006; Ferraro and Vieira, 2010]. In the primary dentition, Shaffer et al. [2015] observed a greater magnitude of the effect of genes in males than females and assumed that different genes might play important roles in each of the sexes in the permanent dentition. Thirdly, we obtained our data using a method based on the TaqMan technique [Koch et al., 2005], while Olszowski et al. [2015] used a method based on PCR previously published by Moleda et al. [2006]. However, some problems with the PCR method with restriction analysis have been reported: in the past a misclassification of ID heterozygotes as DD homozygotes caused by the preferential amplification of the D allele was described in several studies [Shanmugam et al., 1993; Saracevic et al., 2013]. The distribution of the D allele in 41 healthy Polish children in the study by Olszowski et al. [2015] was 68.3%, while another study in the Polish population reported a D allele frequency of only 43.5% in 111 healthy individuals [Zak et al., 2003]. Further, a study by Eleni et al. [2008] summarized that the D allele frequency in healthy subjects varied from 49 to 53% in the European countries. This is in accordance with our results, where the distribution of I/D alleles in 182 healthy children without dental caries was 50:50%.

There are some potential limitations to the present study that need to be considered. Firstly, case-control design carries the risk of providing false-positive results, especially when cases and controls are from different population strata. However, the population studied was homogeneous; children were selected from the ELSPAC cohort and from preschool children of Czech origin from South Moravia. Our study was also relatively large – 8 times bigger than that of Olszowski et al. [2015], therefore the risk of false-negative conclusion was relatively low. Secondly, in multifactorial complex diseases, such as dental caries, each genetic polymorphism has generally only a small effect, and interactions between gene variants and environmental factors can potentially affect the observed phenotype. We focused just on one polymorphism in the *ACE* I/D gene, and did not investigate other susceptibility genes or variants. Thirdly, we did not have bite-wing radiographs, so interproximal lesions may not have been detected. The radiographs, despite their indubitable diagnostic value, were not performed for ethical reasons: to avoid radiation exposure of young children and to reduce to minimum the refusal rate of study participants. Finally, data on circulating levels of ACE protein or its RNA expression were unavailable; therefore, we do not know the functional consequences of this polymorphism in our subjects.

In conclusion, both of the declared aims were fulfilled; variation in the *ACE* gene was examined in the groups of subjects with/without caries in the permanent dentition and also in children with ECC in the primary dentition. Our study suggested that the *ACE* I/D polymorphism might be associated with caries in the permanent but not primary dentition, especially in girls in the Czech population. Nevertheless, the mechanism of a possible ACE effect on susceptibility to dental caries is unknown, and further research in people with different ethnic backgrounds is required.

Downloaded by:
Masaryk University
198.143.55.1 - 2/27/2016 11:45:58 PM

Acknowledgments

The study was supported by grants GACR GB14-37368G and IGA NT11405/6 and projects MUNI/A/1359/2014 and MUNI/M/1075/2013.

Author Contributions

P.B.L., M.K., L.K. and L.I.H. designed the study and drafted the paper. K.M., M.B., L.Z. and M.K. performed the clinical analyses and collected blood samples. J.K. and P.B.L. carried out the molecular analysis. L.I.H. performed the statistical analysis. All authors revised the final version of the manuscript.

Disclosure Statement

The authors declare no conflicts of interest.

References

Bretz WA, Corby P, Hart TC, Costa S, Coelho MQ, Weyant RJ, Robinson M, Schork NJ: Dental caries and microbial acid production in twins. Caries Res 2005;39:168–172.

Bretz WA, Corby P, Schork N, Hart TC: Evidence of a contribution of genetic factors to dental caries risk. J Evid Based Dent Pract 2003;3:185–189.

Eleni S, Dimitrios K, Vaya P, Areti M, Norma V, Magdalini G: Angiotensin-I converting enzyme gene and I/D polymorphism distribution in the Greek population and a comparison with other European populations. J Genet 2008;87:91–93.

European Longitudinal Study of Pregnancy and Childhood (ELSPAC). Paediatr Perinat Epidemiol 1989;3:460–469.

Fejerskov O, Nyvad B, Kidd E: Prologue; in Dental Caries: The Disease and Its Clinical Management, ed 3. Hoboken, Wiley-Blackwell, 2015, p 3.

Ferraro M, Vieira AR: Explaining gender differences in caries: a multifactorial approach to a multifactorial disease. Int J Dent 2010;2010:649643.

Gonzalez-Villalobos RA, Shen XZ, Bernstein EA, Janjulia T, Taylor B, Giani JF, Blackwell WL, Shah KH, Shi PD, Fuchs S, Bernstein KE: Rediscovering ACE: novel insights into the many roles of the angiotensin-converting enzyme. J Mol Med (Berl) 2013;91:1143–1154.

Gürkan A, Emingil G, Saygan BH, Atilla G, Köse T, Baylas H, Berdeli A: Renin-angiotensin gene polymorphisms in relation to severe chronic periodontitis. J Clin Periodontol 2009a;36:204–211.

Gürkan A, Emingil G, Saygan BH, Atilla G, Köse T, Baylas H, Berdeli A: Angiotensin-converting enzyme (ACE), angiotensinogen (AGT), and angiotensin II type 1 receptor (AT1R) gene polymorphisms in generalized aggressive periodontitis. Arch Oral Biol 2009b;54:337–344.

Izakovicova Holla L, Fassmann A, Vasku A, Znojil V, Vanek J, Vacha J: Interactions of lymphotoxin-α (TNF-beta), angiotensin-converting enzyme (ACE), and endothelin-1 (ET-1) gene polymorphisms in adult periodontitis. J Periodontol 2001;72:85–89.

Izakovicova Holla L, Musilova K, Vokurka J, Klapusova L, Pantuckova P, Kukletova M, Kukla L, Znojil V: The association of interleukin-6 (IL-6) haplotypes with plaque-induced gingivitis in children. Acta Odontol Scand 2008;66:105–112.

Kang SW, Han SY, Lim SB, Cho KB, Ban JY: ACE insertion/deletion polymorphism is associated with periodontal disease in Korean population. Arch Oral Biol 2015;60:496–500.

Karakus N, Yigit S, Kalkan G, Sezer S: High association of angiotensin-converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism with recurrent aphthous stomatitis. Arch Dermatol Res 2013;305:513–517.

Koch W, Latz W, Eichinger M, Ganser C, Schömig A, Kastrati A: Genotyping of the angiotensin I-converting enzyme gene insertion/deletion polymorphism by the TaqMan method. Clin Chem 2005;51:1547–1549.

Kukla L, Bouchalova M: European Longitudinal Study of Pregnancy and Childhood (ELSPAC) – Výzkumný projekt WHO/EURO – československá účast (in Czech). Čs Hygiena 1992;37:367–369.

Lukacs JR, Largaespada LL: Explaining sex differences in dental caries prevalence: saliva, hormones, and 'life history' etiologies. Am J Hum Biol 2006;18:540–555.

Ma R, Li X, Su G, Hong Y, Wu X, Wang J, Zhao Z, Song Y, Ma S: Angiotensin-converting enzyme insertion/deletion gene polymorphisms associated with risk of atrial fibrillation: a meta-analysis of 23 case-control studies. J Renin Angiotensin Aldosterone Syst 2015;16:793–800.

Moleda P, Majkowska L, Kaliszczak R, Safranow K, Adler G, Goracy I: Insertion/deletion polymorphism of angiotensin I converting enzyme gene and left ventricular hypertrophy in patients with type 2 diabetes mellitus. Kardiol Pol 2006;64:959–965; discussion 966.

Olszowski T, Adler G, Janiszewska-Olszowska J, Safranow K, Chlubek D: DD Genotype of *ACE* I/D polymorphism might confer protection against dental caries in Polish children. Caries Res 2015;49:390–393.

Olszowksi T, Adler G, Janiszewska-Olszowska J, Safranow K, Kaczmarczyk M: *MBL2*, *MASP2*, *AMELX*, and *ENAM* gene polymorphisms and dental caries in Polish children. Oral Dis 2012;18:389–395.

Ozdemir D: Dental caries: the most common disease worldwide and preventive strategies. International Journal of Biology 2013;5:55–61.

Rahimi Z, Moradi M, Nasri H: A systematic review of the role of renin angiotensin aldosterone system genes in diabetes mellitus, diabetic retinopathy and diabetic neuropathy. J Res Med Sci 2014;19:1090–1098.

Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F: An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. J Clin Invest 1990;86:1343–1346.

Romanos HF, Antunes LS, Lopes LB, Sabóia Tde M, Tannure PN, Lips A, Antunes LA, Abreu FV, Deeley K, Alves G, Granjeiro JM, Vieira AR, Costa MC, Küchler EC: *BMP2* is associated with caries experience in primary teeth. Caries Res 2015;49:425–433.

Saracevic A, Simundic AM, Celap I, Luzanic V: Angiotensin-converting enzyme insertion/deletion polymorphism genotyping error: the cause and a possible solution to the problem. Mol Biol Rep 2013;40:4459–4463.

Shaffer JR, Wang X, McNeil DW, Weyant RJ, Crout R, Marazita ML: Genetic susceptibility to dental caries differs between the sexes: a family-based study. Caries Res 2015;49:133–140.

Shanmugam V, Sell KW, Saha BK: Mistyping ACE heterozygotes. PCR Methods Appl 1993;3:120–121.

Song Y, Miao R, Wang H, Qin X, Zhang Y, Miao C, Wang Z: Meta-analysis of the association between angiotensin-converting enzyme I/D polymorphism and aortic aneurysm risk. J Renin Angiotensin Aldosterone Syst 2015;16:1125–1129.

Downloaded by:
Masaryk University
198.143.55.1 - 2/27/2016 11:45:58 PM

Tiret L, Rigat B, Visvikis S, Breda C, Corvol P, Cambien F, Soubrier F: Evidence, from combined segregation and linkage analysis, that a variant of the angiotensin I-converting enzyme (ACE) gene controls plasma ACE levels. Am J Hum Genet 1992;51:197–205.

Vieira AR, Modesto A, Marazita ML: Caries: review of human genetics research. Caries Res 2014;48:491–506.

Volckova M, Borilova Linhartova P, Trefna T, Vlazny J, Musilova K, Kukletova M, Kukla L, Izakovicova Holla L: Lack of association between lactotransferrin polymorphism and dental caries. Caries Res 2014;48:39–44.

Wang X, Shaffer JR, Weyant RJ, Cuenco KT, DeSensi RS, Crout R, McNeil DW, Marazita ML: Genes and their effects on dental caries may differ between primary and permanent dentitions. Caries Res 2010;44:277–284.

World Health Organisation: Oral Health Surveys: Basic Methods, ed 4. Geneva, WHO, 1997.

Yang J, Zhao Y, Hao P, Meng X, Dong M, Wang Y, Zhang Y, Zhang C: Impact of angiotensin I converting enzyme insertion/deletion polymorphisms on dilated cardiomyopathy and hypertrophic cardiomyopathy risk. PLoS One 2013;8:e63309.

Yu ZY, Chen LS, Zhang LC, Zhou TB: Meta-analysis of the relationship between *ACE* I/D gene polymorphism and end-stage renal disease in patients with diabetic nephropathy. Nephrology (Carlton) 2012;17:480–487.

Zak I, Niemiec P, Sarecka B, Balcerzyk A, Ciemniewski Z, Rudowska E, Dylag S: Carrier-state of D allele in *ACE* gene insertion/deletion polymorphism is associated with coronary artery disease, in contrast to the C677–>T transition in the *MTHFR* gene. Acta Biochim Pol 2003;50:527–534.

Zhang K, Cheng D, Yi L, Shi H, Zhen G: Association between angiotensin I-converting enzyme gene polymorphism and susceptibility to cancer: a meta-analysis. Int J Clin Exp Pathol 2014;7:6291–6300.

Zhao J, Qin X, Li S, Zeng Z: Association between the *ACE* I/D polymorphism and risk of ischemic stroke: an updated meta-analysis of 47,026 subjects from 105 case-control studies. J Neurol Sci 2014;345:37–47.

Downloaded by:
Masaryk University
195.113.55.1 - 2/27/2016 11:45:56 PM

3.2 Comment to the article ”Vitamin D receptor *Taq*I gene polymorphism and dental caries in Czech children”

Izakovicova Holla L, Borilova Linhartova P, Kastovsky J, Bartosova M, Musilova K, Kukla L, Kukletova M. Vitamin D receptor *Taq*I gene polymorphism and dental caries in Czech children. Caries Res. 2017;51(1):7-11. (IF 2.188; DENTISTRY, ORAL SURGERY & MEDICINE Q2)

Author contribution: P.B.L. particularly designed the study, drafted the paper, and carried out genetic analysis.

Vitamin D is not only a regulator of mineral homeostasis through the mediation of calcium absorption which can influence the quality of bone, enamel, and dentin, but it is also involved in the immune response (Lin, 2016). The biologically most active metabolite of vitamin D, $1,25(OH)_2D_3$, acts by binding an intracellular vitamin D receptor (VDR). The gene encoding the VDR contains *Taq*I polymorphism which was associated with increased transcriptional activity, mRNA stability, and a high serum level of $1,25(OH)_2D_3$ (Martelli et al., 2014).

The aims of our study were to (i) examine the associations between this VDR variant and dental caries in Czech children with permanent dentition, (ii) compare the *VDR Taq*I allele and genotype distributions between subgroups with mild/moderate or high caries experience, and (iii) investigate the impact of this polymorphism on gingivitis susceptibility.

A total of 388 subjects from ELSPAC, 235 children with dental caries (DMFT≥1) and 153 caries-free (DMFT=0) children were genotyped using the TaqMan method for *VDR Taq*I rs731236 polymorphism.

Although no significant associations between the *VDR Taq*I polymorphism and caries susceptibility and/or severity were found, statistically significant differences were observed between children with gingivitis (gingival index, GI>0) and those with healthy gingiva (GI=0). The *VDR Taq*I T allele and TT genotype were more frequently present in children with gingivitis ($P<0.05$, OR=1.66, 95%CI=1.23-2.26, and $P<0.05$, OR=2.13, 95%CI=1.09-4.16, respectively).

Several studies have evaluated the potential association between this *VDR* gene polymorphism and an increased susceptibility to periodontitis (Deng et al., 2011). Before our study, only 2 studies had analyzed allele and genotype frequencies of the *VDR Taq*I polymorphism in patients with dental caries (Hu et al., 2015; Cogulu et al., 2016).

In contrast to previous studies from China and Turkey (Hu et al., 2015; Cogulu et al., 2016), the *VDR Taq*I gene variant cannot be used as a marker for the identification of Czech children

with an increased risk of dental caries. The differences in the association of the *VDR Taq*I polymorphism with caries between studies can be considered in relation to the selection of inclusion criteria (children with mixed dentition were included in the Turkish study (Cogulu et al., 2016), sample size, ethnic background, and thus differences in MAF. In addition, no association of this SNP with dental caries was found in the later Chinese work in children with primary dentition (Kong et al., 2017).

Short Communication

Caries Research

Caries Res 2017;51:7–11
DOI: 10.1159/000452635

Received: September 1, 2016
Accepted after revision: October 17, 2016
Published online: November 26, 2016

Vitamin D Receptor *Taq*I Gene Polymorphism and Dental Caries in Czech Children

Lydie Izakovicova Holla[a, b] Petra Borilova Linhartova[a, b] Jakub Kastovsky[a, b] Michaela Bartosova[a, c] Kristina Musilova[a] Lubomir Kukla[c] Martina Kukletova[a]

[a]Clinic of Stomatology, Institutions Shared with St. Anne's Faculty Hospital, and [b]Department of Pathophysiology, Faculty of Medicine, and [c]Research Centre for Toxic Compounds in the Environment (RECETOX), Chemistry Section, Faculty of Science, Masaryk University, Brno, Czech Republic

Keywords

Case-control study · Dental caries · Gene polymorphism · Gingivitis · Vitamin D receptor

Abstract

Aim: We analyzed the *VDR TaqI* (rs731236) gene polymorphism in children with and those without dental caries. ***Methods:*** A total of 388 subjects, 153 caries-free (with decayed/missing/filled teeth [DMFT] = 0) and 235 children with dental caries (DMFT ≥1), were genotyped by the TaqMan method. ***Results:*** Although no significant differences in *VDR TaqI* allele and genotype frequencies between caries-free and caries-affected children were detected, a significant association between this polymorphism and gingivitis was found ($p < 0.05$). ***Conclusions:*** In contrast to previous studies from China and Turkey, the *VDR TaqI* gene variant cannot be used as a marker for identification of Czech children with increased dental caries risk.

© 2016 S. Karger AG, Basel

Dental caries is one of the most frequent multifactorial diseases and affects 60–90% of the population [Ozdemir, 2013]. The etiology of caries includes environmental factors such as dental plaque with cariogenic bacteria, diet with high contents of carbohydrates, poor oral hygiene, low fluoride exposure, and inadequate saliva flow. However, when exposed to the same level of environmental factors, some patients may be more susceptible to caries than others [Yildiz et al., 2016]. There are strong arguments for genetic contribution in dental caries, and several candidate genes may be related to the structure of enamel, composition of saliva, and host inflammatory response to cariogenic bacteria [Vieira et al., 2014].

Vitamin D is not only a regulator of mineral homeostasis through the mediation of calcium absorption which can influence the quality of bone, enamel, and dentin, but it is also involved in immune response [Lin, 2016]. The biologically most active metabolite of vitamin D, $1,25(OH)_2D_3$, acts by binding an intracellular vitamin D receptor (VDR). The gene encoding the VDR, located on the chromosome 12q13.11, contains several polymorphic regions. The *Taq*I polymorphism that is characterized by a single base transition (T>C) leading to a synonymous change at codon 352 in exon 9 belongs to the most studied variant. It creates a *Taq*I restriction site; resulting alleles are called "T" (*Taq*I site absent) and "t" (*Taq*I site present). The presence of the "t" allele correlates with in-

KARGER

© 2016 S. Karger AG, Basel

E-Mail karger@karger.com
www.karger.com/cre

Prof. Lydie Izakovicova Holla, MD, PhD
Clinic of Stomatology and Department of Pathophysiology
Faculty of Medicine, Masaryk University
Pekarska 664/53, CZ–602 00 Brno (Czech Republic)
E-Mail holla@med.muni.cz

Downloaded by:
Masaryk University
147.251.139.16 - 12/7/2016 7:21:53 AM

creased transcriptional activity, mRNA stability, and a high serum level of 1,25$(OH)_2D_3$ [Martelli et al., 2014]. Several studies evaluated the potential association between this *VDR* gene polymorphism and an increased susceptibility to periodontitis [Deng et al., 2011]. To date, only 2 studies have analyzed allele and genotype frequencies of the *VDR TaqI* polymorphism in patients with dental caries in Turkish and Chinese populations [Hu et al., 2015; Cogulu et al., 2016]. However, no study has been conducted on Caucasians yet.

Therefore, in the present study we aimed to (a) examine the associations between this *VDR* variant and dental caries in Czech children, (b) compare the *VDR TaqI* allele and genotype distributions between subgroups with mild/moderate or high caries experience, and (c) investigate the impact of this polymorphism on gingivitis susceptibility.

Methods

Subjects

A total of 388 unrelated Caucasian children (203 boys and 185 girls), aged 13–15 years, selected from the prospective European Longitudinal Study of Pregnancy and Childhood (ELSPAC) Brno study comprising over 7,000 children and their families [ELSPAC, 1989; Piler et al., 2016], were investigated. Of the whole ELSPAC set, 900 individuals were randomly selected and invited to participate in the study of oral health. Parents of 780 adolescents agreed, and during 2005–2007 these children underwent a detailed dental examination. The *VDR TaqI* gene polymorphism was analyzed in 388 randomly chosen subjects in this preliminary study. The inclusion criteria consisted of simple informed consent of the children and their parents and an expression of their willingness to participate. The children underwent dental examination at the Clinic of Stomatology at St. Anne's University Hospital and the Faculty of Medicine, Masaryk University, as described previously [Volckova et al., 2014]. The DMFT score was calculated according to guidelines of the World Health Organization (1997), the gingival index (GI), the plaque index, and the calculus index, as published in our previous study [Izakovicova Holla et al., 2008].

The study was approved by the Committee for Ethics of the Faculty of Medicine, Masaryk University Brno (3/2004, from 30/03/2004), and St. Anne's Faculty Hospital (without number, from 13/04/2004) and the informed consent was obtained from all parents (in the case of children), in line with the Declaration of Helsinki, prior to their inclusion in the study.

SNPs Genotyping TaqMan® Assay

DNA for genetic analysis was isolated from buccal epithelial cells. Extractions were performed according to the manufacturer's instructions for the UltraClean® BloodSpin® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) with small adaptations.

Genotyping of SNP *TaqI* in the *VDR* gene (rs731236, +61968T/C) was based on polymerase chain reaction using a 5′ nuclease TaqMan® assay (C_2404008_10). The reaction mixture and conditions were designed according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA), and fluorescence was measured using the ABI PRISM 7000 Sequence Detection System. SDS version 1.2.3 software was used to analyze real-time and endpoint fluorescence data. Genotyping was verified by using positive control subjects in each 96-well plate and rerunning ≥5% of the samples, which were 100% concordant.

Statistical Analysis

Standard descriptive statistics were applied in the analysis: mean and standard deviation for quantitative variables and absolute and relative frequencies for categorical variables. The χ^2 and Fisher exact tests were used for comparison of differences in genotypes or allele frequencies between groups. Odds ratios, confidence intervals, and p values were calculated. Power analysis was performed with respect to the case-control design of the study. All statistical analyses were performed using the program package Statistica version 12 (StatSoft Inc., Tulsa, OK, USA) and SPSS software (SPSS 22; IBM Corporation, 2013).

Results

The study group comprised caries-free (DMFT = 0; $n = 153$; 81 boys and 72 girls) and caries-affected subjects (DMFT ≥1; $n = 235$; 122 boys and 113 girls); 95 (40.0%) of the caries-affected children were classified as having low/moderate caries experience (1 ≤ DMFT ≤ 4) and 140 (60.0%) as high caries experience (DMFT > 4). Healthy gingiva (GI = 0) as the highest score was found in 38.9%, grade 1 in 44.8%, and grade 2 in 16.2% of the children; grade 3 was not detected. The mean GI value per child was 0.23 (SE = 0.016). The mean values of the plaque index and the calculus index were 0.67 and 0.65, respectively. Power calculation was used to estimate statistically detectable differences (effect size). With standard statistical procedures applied (power 80%, α error 5%), a sample size of 388 subjects was sufficient to detect significant differences ≥15% in the relative rate of a *TaqI* marker between the subgroups.

There were no significant differences in the *VDR TaqI* genotype distributions and allele frequencies between the groups of children with and those without caries, between caries-free subjects and subjects with high caries experience, or between patients with mild/moderate and those high caries experience, as shown in Table 1.

Although no significant associations between the *VDR TaqI* polymorphism and caries susceptibility and/or severity were found, statistically significant differences were observed between children with healthy gingiva (GI = 0) and those with gingivitis (GI > 0). The T allele and TT genotype were more frequently present in children with gingivitis ($p = 0.016$ and $p < 0.05$, respectively, see Table 2).

Downloaded by:
Masaryk University
147.251.139.16 - 12/7/2016 7:21:59 AM

Table 1. Distribution of the *VDR TaqI* (rs731236) genotype and allele frequencies in children with caries experience (DMFT ≥ 1), mild/moderate (1 ≤ DMFT ≤ 4), and high (DMFT > 4) caries experience versus caries-free children (DMFT = 0)

	Caries-free children (DMFT = 0)	Caries-affected children (DMFT ≥ 1)	Mild/moderate caries experience (1 ≤ DMFT ≤ 4)	High caries experience (DMFT > 4)
VDR TaqI (rs731236)	153 (100.0)	235 (100.0)	95 (100.0)	140 (100.0)
TT	51 (33.3)	95 (40.4)	38 (40.0)	57 (40.7)
Tt	85 (55.6)	110 (46.8)	47 (49.5)	63 (45.0)
tt	17 (11.1)	30 (12.8)	10 (10.5)	20 (14.3)
T allele	187 (61.1)	300 (63.8)	123 (64.7)	177 (63.2)
t allele	119 (38.9)	170 (36.2)	67 (35.3)	103 (36.8)

Values represent *n* (%) of subjects. VDR, vitamin D receptor; DMFT, decayed/missing/filled teeth.

Table 2. Distribution of the *VDR TaqI* (rs731236) genotype and allele frequencies in children with gingivitis (GI > 0) and controls with healthy gingiva (GI = 0)

	Children with healthy gingiva (GI = 0)	Children with gingivitis (GI > 0)	OR (95% CI)
VDR TaqI (rs731236)	151 (100.0)	237 (100.0)	
TT	48 (31.8)	98 (41.4)*	2.13 (1.09–4.16)
Tt	79 (52.3)	116 (48.9)	1.53 (0.81–2.90)
tt	24 (15.9)	23 (9.7)	1.00
T allele	175 (57.9)	312 (65.8)*	1.66 (1.23–2.26)
t allele	127 (42.1)	162 (34.2)	1.00

Values represent *n* (%) of subjects. VDR, vitamin D receptor; CI, confidence interval; GI, gingival index; OR, odds ratio. * $p < 0.05$.

Discussion

Vitamin D plays a role in maintaining oral health through its effects on bone and mineral metabolism and its immunomodulatory and anti-inflammatory properties [von Essen et al., 2010]. VDR is the mediator of the vitamin D pleiotropic biological actions. In this study, we evaluated the *TaqI* polymorphism of the *VDR* gene in a group of Czech children aged 13–15 years. Our data showed no significant differences in frequencies of alleles or genotypes of the *TaqI* variant between caries-free and caries-affected children or those with different levels of dental caries experience. These findings are in disagreement with results published by Hu et al. [2015], who suggested that the "t" allele and "Tt" genotype might be risk factors of susceptibility to dental caries in adults from the northwest of China. Further, the *VDR TaqI* "tt" genotype was very recently reported to increase the risk of caries in Turkish children [Cogulu et al., 2016]. In contrast to European (Czech) subjects, where the frequency of "T" versus "t" alleles was 61 versus 39% in healthy children, in the Chinese population the "T" allele was the dominant allele, with a frequency of 95.7%, and no "tt" homozygotes were observed in a group of 483 adult people [Hu et al., 2015]. In a small group of 38 caries-free children aged 6–12 years from Turkey, the frequency of "T" versus "t" alleles was 58 versus 42%, and the authors associated only the "tt" genotype with high caries risk [Cogulu et al., 2016]. In contrast to our study, their children had mixed (not only permanent) dentition; there are some findings that genes affecting susceptibility to caries can differ between the 2 types of teeth [Wang et al., 2010; Borilova Linhartova et al., 2016]. The differences in the association of the *VDR TaqI* polymorphism with caries between studies can be considered in relation to the methodological approaches applied, sample size, and ethnic background. Firstly, both previous studies used a method based on PCR for the determination of this *VDR* variant,

Downloaded by:
Masaryk University
147.251.139.16 - 12/7/2016 7:21:53 AM

with following *Taq*I restriction enzyme analysis. In contrast, our data were obtained by the TaqMan technique, which is considered to be more accurate. Secondly, a sample size is critical for case-control studies. Cogulu et al. [2016] examined 350 children aged 6–12 years, but a final association between the *VDR Taq*I polymorphism and caries was found only in a group of 150 children (75 boys and 75 girls) with similar dental plaque scores, hygiene habits, and sugar consumption, which was further divided into 3 small subgroups of caries-free, moderate, and high caries ($n = 38, 57$, and 55, respectively). However, we analyzed the study population of 388 unrelated children at the age of 13–15 years, which was more than twice the size of the Turkish study [Cogulu et al., 2016] and only a little smaller than the first study by Hu et al. [2015]. Thirdly, different genetic backgrounds due to different ethnicity (Asians vs. Caucasians) in the 3 studies could be a cause of heterogeneity of the results. Apart from the ethnic variations, geographical differences and the interaction between *VDR* gene variants and environmental conditions may also differ between populations [Papadopoulou et al., 2015].

The aim of the third experiment was to assess the *VDR Taq*I polymorphism in groups according to gingival health status. We found that the "T" allele and "TT" genotype were associated with an increased risk of gingivitis. Our results are in agreement with previous findings suggesting that the "TT" genotype and the presence of the "T" allele were associated with chronic periodontal disease in Japanese, Chinese, and Caucasian subjects [Martelli et al., 2014] and also with aggressive periodontal disease [Martelli et al., 2011]. In addition, a meta-analysis of 15 studies including 1,338 cases and 1,302 controls suggested a weak but significantly higher frequency of the *Taq*I "TT" genotype in chronic periodontitis [Deng et al., 2011]. Functionally, the *VDR* "TT" genotype has been correlated with lower serum levels of vitamin $1,25(OH)_2D_3$ [Morrison et al., 1994] and increased circulating reactive proteins [Timms et al., 2002]; this may explain a higher susceptibility to inflammation. In contrast, there are also some studies that found an association between the carriage of the less frequent "t" allele and periodontitis [Sun et al., 2002; Kaarthikeyan et al., 2013] or no differences in the *VDR Taq*I polymorphism between healthy subjects and patients with periodontal diseases [Chantarangsu et al., 2016].

There are some limitations of this study that need to be considered. The case-control approach used is vulnerable to the population stratification, e.g., ethnic origin. However, our children were exclusively of Czech Caucasian origin living in a limited geographical region (Brno). The next complicating factor is that we studied only 1 polymorphism of the *VDR* gene in multifactorial disease (caries or gingivitis) where interactions of multiple genes and environmental factors are assumed. Finally, we have no information regarding the presence of *Streptococcus mutans*, a major bacterium connected with increased risk of caries.

In summary, although the results of 2 previous studies indicated that *Taq*I variants of the *VDR* gene might be associated with dental caries [Hu et al., 2015; Cogulu et al., 2016], the findings of this study suggest that this polymorphism is likely to contribute to gingival status in the Czech population. Nevertheless, further investigations are needed to confirm this finding in larger populations with different ethnic backgrounds.

Acknowledgments

The study was supported by funds from the Faculty of Medicine MU to junior researcher Petra Borilova Linhartova, grant GACR GB14-37368G and project MUNI/A/1258/2015. We thank Assoc. Prof. Ladislav Dusek, PhD, head of the Institute of Biostatistics and Analyses, Masaryk University, Brno, Czech Republic, for the calculation of power analysis.

Author Contributions

L.I.H, M.K., L.K., and P.B.L. designed the study and drafted the paper. K.M., M.B., L.K., and M.K. performed the clinical analyses and collected saliva samples. J.K. and P.B.L. carried out the molecular analysis. L.I.H. performed the statistical analysis. All authors revised the final version of the manuscript.

Disclosure Statement

The authors declare no conflicts of interest.

References

Borilova Linhartova P, Kastovsky J, Bartosova M, Musilova K, Zackova L, Kukletova M, Kukla L, Izakovicova Holla L: ACE I/D polymorphism associated with caries in permanent but not primary dentition in Czech children. Caries Res 2016;50:89–96.

Chantarangsu S, Sura T, Mongkornkarn S, Donsakul K, Torrungruang K: Vitamin D receptor gene polymorphism and smoking in the risk of chronic periodontitis. J Periodontol 2016; 87:1343–1351.

Downloaded by:
Masaryk University
147.251.139.16 - 12/7/2016 7:21:59 AM

- Cogulu D, Onay H, Ozdemir Y, Aslan GI, Ozkinay F, Eronat C: The role of vitamin D receptor polymorphisms on dental caries. J Clin Pediatr Dent 2016;40:211–214.
- Deng H, Liu F, Pan Y, Jin X, Wang H, Cao J: *Bsm*I, *Taq*I, *Apa*I, and *Fok*I polymorphisms in the vitamin D receptor gene and periodontitis: a meta-analysis of 15 studies including 1,338 cases and 1,302 controls. J Clin Periodontol 2011;38:199–207.
- European Longitudinal Study of Pregnancy and Childhood (ELSPAC). Paediatr Perinat Epidemiol 1989;3:460–469.
- Hu XP, Li ZQ, Zhou JY, Yu ZH, Zhang JM, Guo ML: Analysis of the association between polymorphisms in the vitamin D receptor (VDR) gene and dental caries in a Chinese population. Genet Mol Res 2015;14:11631–11638.
- Izakovicova Holla L, Musilova K, Vokurka J, Klapusova L, Pantuckova P, Kukletova M, Kukla L, Znojil V: The association of interleukin-6 (IL-6) haplotypes with plaque-induced gingivitis in children. Acta Odontol Scand 2008;66:105–112.
- Kaarthikeyan G, Jayakumar ND, Padmalatha O, Varghese S, Anand B: Analysis of association of TaqI VDR gene polymorphism with the chronic periodontitis in Dravidian ethnicity. Indian J Hum Genet 2013;19:465–468.
- Lin R: Crosstalk between vitamin D metabolism, VDR signalling, and innate immunity. Biomed Res Int 2016;2016:1375858.
- Martelli FS, Martelli M, Rosati C, Fanti E: Vitamin D: relevance in dental practice. Clin Cases Miner Bone Metab 2014;11:15–19.
- Martelli FS, Mengoni A, Martelli M, Rosati C, Fanti E: VDR *Taq*I polymorphism is associated with chronic periodontitis in Italian population. Arch Oral Biol 2011;56:1494–1498.
- Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA: Prediction of bone density from vitamin D receptor alleles. Nature 1994;367:284–287.
- Ozdemir D: Dental caries: the most common disease worldwide and preventive strategies. Int J Biol 2013;5:55–61.
- Papadopoulou A, Kouis P, Middleton N, Kolokotroni O, Karpathios T, Nicolaidou P, Yiallouros PK: Association of vitamin D receptor gene polymorphisms and vitamin D levels with asthma and atopy in Cypriot adolescents: a case-control study. Multidiscip Respir Med 2015;10:26.
- Piler P, Kandrnal V, Kukla L, Andrýsková L, Švancara J, Jarkovský J, Dušek L, Pikhart H, Bobák M, Klánová J: Cohort Profile: The European Longitudinal Study of Pregnancy and Childhood (ELSPAC) in the Czech Republic. Int J Epidemiol 2016, Epub ahead of print.
- Sun JL, Meng HX, Cao CF, Tachi Y, Shinohara M, Ueda M, Imai H, Ohura K: Relationship between vitamin D receptor gene polymorphism and periodontitis. J Periodontal Res 2002;37:263–267.
- Timms PM, Mannan N, Hitman GA, Noonan K, Mills PG, Syndercombe-Court D, Aganna E, Price CP, Boucher BJ: Circulating MMP9, vitamin D and variation in the TIMP-1 response with VDR genotype: mechanisms for inflammatory damage in chronic disorders? QJM 2002;95:787–796.
- Vieira AR, Modesto A, Marazita ML: Caries: review of human genetics research. Caries Res 2014;48:491–506.
- Volckova M, Borilova Linhartova P, Trefna T, Vlazny J, Musilova K, Kukletova M, Kukla L, Izakovicova Holla L: Lack of association between lactotransferrin polymorphism and dental caries. Caries Res 2014;48:19–44.
- von Essen MR, Kongsbak M, Schjerling P, Olgaard K, Ødum N, Geisler C: Vitamin D controls T cell antigen receptor signaling and activation of human T cells. Nat Immunol 2010;11:344–349.
- Wang X, Shaffer JR, Weyant RJ, Cuenco KT, DeSensi RS, Crout R, McNeil DW, Marazita ML: Genes and their effects on dental caries may differ between primary and permanent dentitions. Caries Res 2010;44:277–284.
- Yildiz G, Ermis RB, Calapoglu NS, Celik EU, Türel GY: Gene-environment interactions in the etiology of dental caries. J Dent Res 2016;95:74–79.

Downloaded by:
Masaryk University
147.251.139.16 - 12/7/2016 7:21:59 AM

4. Periodontitis

Note: This chapter builds on my Dissertation thesis (Bořilová Linhartová, 2014).

Periodontitis has the effect loss of connective tissues, alveolar bone and eventually teeth. There is strong evidence for an association between an peridontitis and diabetes mellitus (DM). Between both diseases is a reciprocal relationship, which has been intensively studied. In addition, the some risk factors for dental caries are either more or less prevalent in the diabetic population (Novotna et al., 2015).

DM increases the risk for and severity of periodontitis, and periodontal diseases can aggravate insulin resistance and affect glycemic control (Stanko and Izakovicova Holla, 2014). Apart from the systemic effects of DM, recent evidence suggests that local changes in the periodontal tissues are characterized by enhanced interactions between leukocytes and endothelial cells and altered leukocyte functions (Sonnenschein and Meyle, 2015). Increased levels of ROS (Yan, 2014) and pro-inflammatory cytokines – lead to chronic low-grade inflammation, change of cytokine spectrum, and activation of innate immunity (Odegaard and Chawla, 2012).

The main aim of the our recent clinical study (Poskerová et al., 2018, Attachment 11) was to investigate the state of periodontium in Czech diabetic patients. Three groups of subjects: T1DM and T2DM patients, and subjects without DM (controls) were compared. The subjects underwent clinical and radiological examination during years 2010–2015 and 7 selected periodontal bacteria (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannarella forsythia*, *Treponema denticola*, *Parvimonas micra*, *Prevotella intermedia*, *Fusobacterium nucleatum*) were analyzed in their subgingival plaque by a DNA microarray detection kit in external laboratory (Stomagene, České Budějovice, Czech Republic).

The study comprised a total of 141 subjects (32 with T1DM, 51 with T2DM, 58 controls) aged 35–65 years. The state of teeth and periodontium in diabetic (mainly T2DM) patients was worse than in controls. This finding was confirmed for plaque index (PI) and GI, number of extracted teeth, the presence of teeth replacement and other parameters evaluating the presence and severity of periodontitis ($P<0.05$, see Fig. 12). Patients with T2DM and diabetic nephropathy had worse state of the periodontium; this finding was not proven in patients with insufficiently controlled diabetes or the disease duration ($P<0.05$).

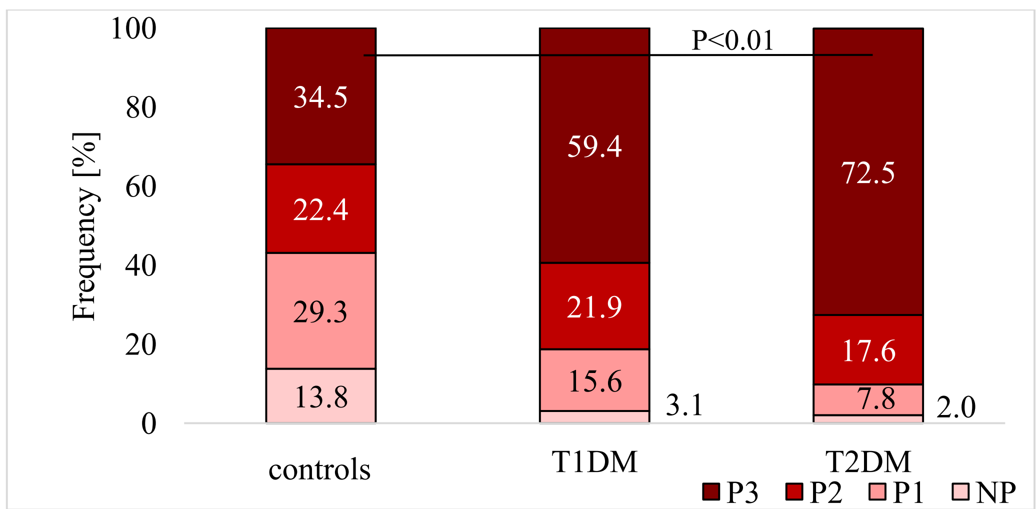
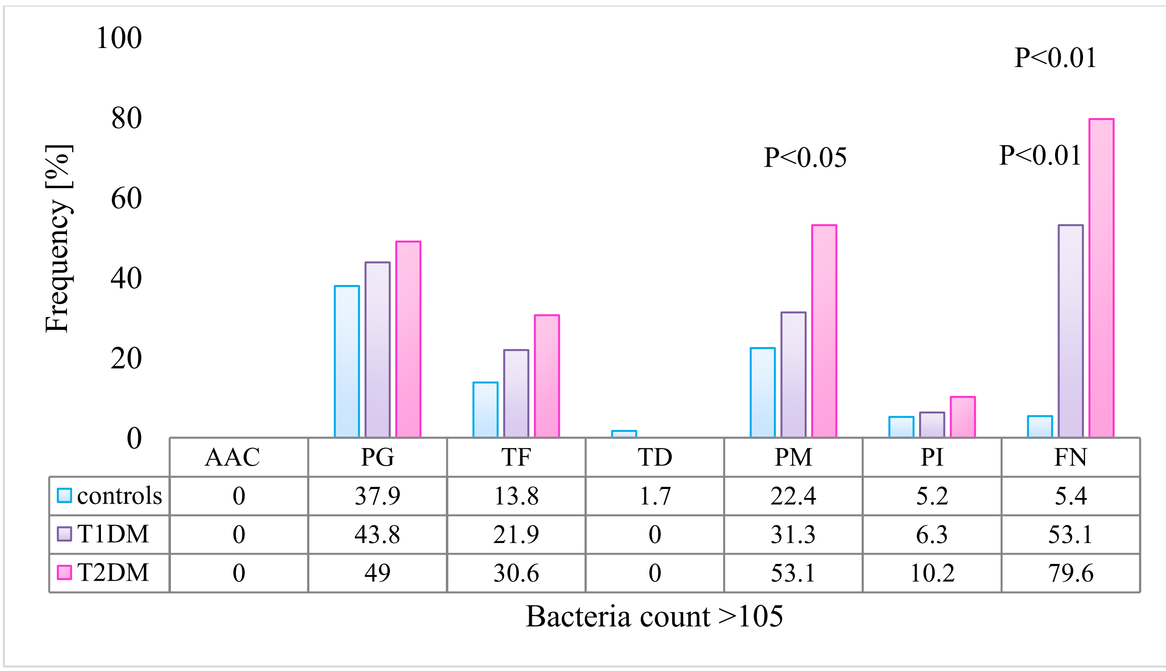


Fig. 12. Comparison of the prevalence and severity of periodontitis in diabetic patients and in the controls (Poskerová et al., 2018).

NP=non-periodontitis, P1/2/3=mild/moderate/severe periodontitis

The differences in the representation of periodontal bacteria and their quantity among the groups were minimal, only *P. micra* and *F. nucleatum* occurred more often in subgingival plaque from diabetics than from controls ((P<0.05 and P<0.01, respectively, see Fig. 13).



	AAC	PG	TF	TD	PM	PI	FN
controls	0	37.9	13.8	1.7	22.4	5.2	5.4
T1DM	0	43.8	21.9	0	31.3	6.3	53.1
T2DM	0	49	30.6	0	53.1	10.2	79.6

Fig. 13. Comparison of subgingival microflora in diabetic patients and in controls (Poskerová et al., 2018).

AAC=*Aggregatibacter actinomycetemcomitans*, PG=*Porphyromonas gingivalis*, TN=*Tannarella forsythia*, TD=*Treponema denticola*, PM=*Parvimonas micra*, PI=*Prevotella intermedia*, FN=*Fusobacterium nucleatum*

In conclusion, diabetic patients suffered more often by inflammatory periodontal diseases and other diseases of the oral cavity than controls. The relationship between DM and oral tissue diseases were summarized in our recent review (Poskerová et al., 2019, Attachment 12).

Besides environmental factors of stress, smoking, obesity etc., plays the role genetic predispositions in the etiopathogenesis of both diseases. Candidate genes for periodontitis are these encoding immunoregulatory factors, especially cytokines (Fig. 14). Overview by Heidari et al. (2019) contains findings of studies dealing with immunomodulatory factors gene polymorphisms in CP. In the etiopathogenesis of T1DM and/or T2DM play a role among others IL-1, IL-8 and IL-17 (Dakovic et al., 2013; Liu et al., 2017; Abdel-Moneim et al., 2018).

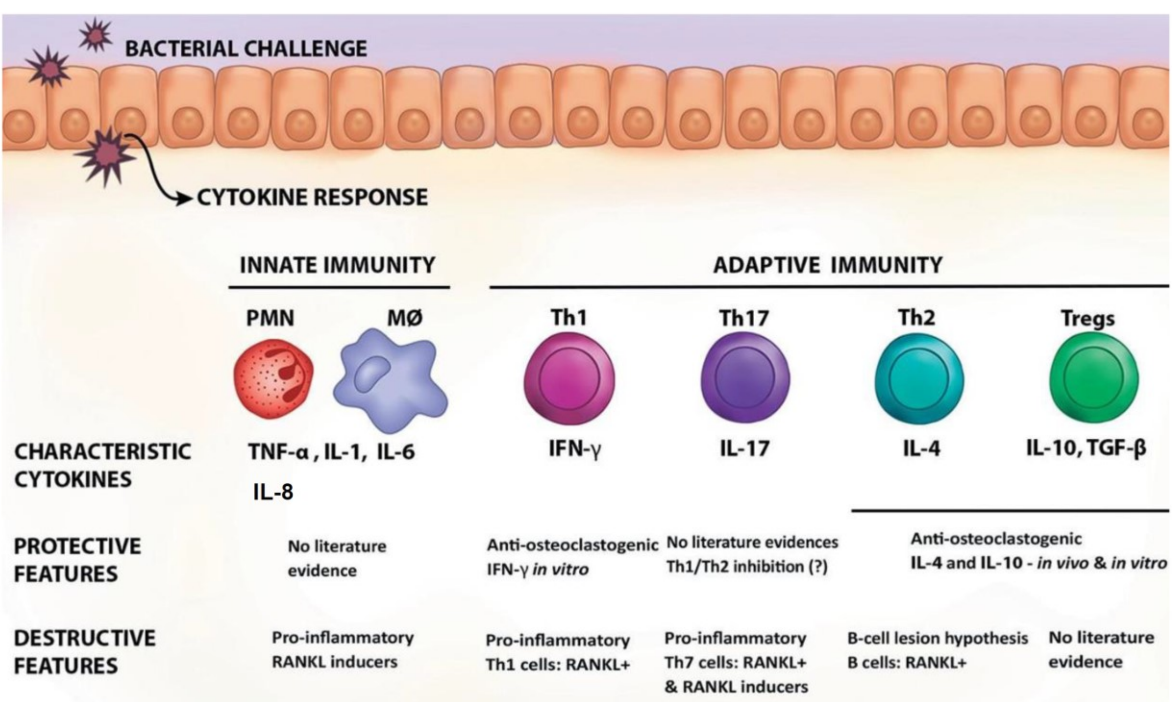


Fig. 14. Cytokines and periodontal disease – sligthly modified (Silva et al., 2015).

4.1 Comment to the article ”Interleukin-1 gene variability and plasma levels in Czech patients with chronic periodontitis and diabetes mellitus”

Borilova Linhartova P, Poskerova H, Tomandlova M, Bartova J, Kankova K, Fassmann A, Izakovicova Holla L. Interleukin-1 gene variability and plasma levels in Czech patients with chronic periodontitis and diabetes mellitus. Int J Dent. 2019;2019:6802349. DOI: 10.1155/2019/6802349.

Author contribution: P.B.L. designed the study, drafted the paper, and carried out the genetic analysis.

The current study was based on our pilot work dealing with polymorphisms in selected cytokines, specifically in the *IL-1* gene cluster and the *IL-6* gene, in patients with CP with/without DM and in healthy subjects (Bořilová Linhartová et al., 2015, Attachment 13). The first aim of this study was to investigate two SNPs and one VNTR polymorphism in the *IL-1* gene cluster in CP patients, diabetic patients of both types (T1DM and T2DM), and non-periodontitis systemically healthy controls (HC) in the Czech population. The second objective was to compare the subset of diabetic patients with CP and generally healthy patients with/without CP or diabetic patients according to their *IL-1* haplotype profile. Finally, assuming higher circulating IL-1β levels in diabetic patients with CP than in nondiabetic patients with CP, the plasma levels in these groups of patients and their correlation to polymorphisms in the *IL-1* gene cluster were analyzed. In addition, we examined the IL-1β levels in mononuclear cells of generally healthy subjects after stimulation by oral pathogens, mitogens, or heat shock protein 70 (HSP70).
A total of 1016 individuals participated in this case-control study. DNA from 264 patients with CP, 132 with T1DM, 395 patients with T2DM, and 225 non-periodontitis HC were genotyped using methods based on PCR techniques for *IL-1* gene polymorphisms (*IL-1A* rs1800587, *IL-1B* rs1143634, and *IL-1RN* 86 bp tandem repeats in intron 2). The levels of IL-1β were measured using Luminex methods in subgroups of CP, T1DM+CP, and T2DM CP subjects, and HC. Isolation, cultivation and stimulation of peripheral blood mononuclear cells (PBMCs) by selected periodontal bacteria (*A. actinomycetemcomitans, P. gingivalis*, *P. intermedia*, and *T. forsythia*), mitogens, or HSP70 were described in our previous study (Bartova et al., 2014).
Although no significant associations were found in the genotype and allele frequencies of *IL-1A* rs1800587 ($P>0.05$), the *IL-1B* rs1143634 T allele was less frequent in CP patients than in the non-periodontitis HC ($P<0.05$, OR=0.70, 95%CI=0.52-0.94). In T1DM patients,

the *IL-1RN**S "short" allele and the *IL-1RN* 12 genotype were significantly less frequent than those in the non-periodontitis HC (P<0.01, OR=0.64, 95%CI=0.44-0.92, and P<0.01, OR=0.46, 95%CI=0.28-0.76, respectively). In haplotype analysis, the *IL-1* gene cluster TTL haplotype decreased the risk of CP development (P<0.01, OR=0.64, 95%CI=0.47-0.88), whereas CCS and CTL haplotypes were associated with T1DM (P<0.01, OR=0.58, 95%CI=0.39-0.87, and P<0.05, OR=1.98, 95%CI=1.01-3.87, respectively).

No significant differences in IL-1β plasma levels in diabetic patients and the HC with similar periodontal conditions were found (P>0.05), *IL-1* genotypes did not correlate with circulating IL-1β levels (P>0.05). Highly significant differences were observed in IL-1β levels between unstimulated PBMCs and cells after stimulation by Pokeweed mitogen (PWM), PWM in costimulation by Concavalin A (Con A), selected periodontal bacteria (*A. actinomycetemcomitans*, *P. intermedia*, and *T. forsythia*) (all P<0.001), or HSP70 (P<0.05).

To our knowledge, this is the first study comparing IL-1β plasma levels in T2DM patients with CP and nondiabetic patients with CP. Although we assumed significant higher concentrations of this cytokine in all diabetic patients than in generally healthy patients with similar periodontal conditions, only a slightly elevated IL-1β levels were found in T1DM+CP or T2DM+CP patients compared with the CP subjects (median 3.26 $pgmL^{-1}$ or 3.87 $pgmL^{-1}$ vs. 2.57 $pgmL^{-1}$).

In the Czech population, significant associations between the *IL-1B* polymorphism with CP and the *IL-1RN* variant with T1DM were found. Haplotype analysis suggests that variability in the *IL-1* gene cluster may be one of the factors in the CP and T1DM pathogenesis, although single variants of these polymorphisms are not substantial for protein production.

Hindawi
International Journal of Dentistry
Volume 2019, Article ID 6802349, 10 pages
https://doi.org/10.1155/2019/6802349

Research Article

Interleukin-1 Gene Variability and Plasma Levels in Czech Patients with Chronic Periodontitis and Diabetes Mellitus

Petra Borilova Linhartova [1,2] **Hana Poskerova** [1] **Marie Tomandlova,** [3] **Jirina Bartova** [4] **Katerina Kankova** [2] **Antonin Fassmann,** [1] **and Lydie Izakovicova Holla** [1,2]

[1] *Clinic of Stomatology, Institutions Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Brno 60200, Czech Republic*
[2] *Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno 62500, Czech Republic*
[3] *Department of Biochemistry, Faculty of Medicine, Masaryk University, Brno 62500, Czech Republic*
[4] *Institute of Clinical and Experimental Dental Medicine, General University Hospital and First Faculty of Medicine, Charles University, Prague 12000, Czech Republic*

Correspondence should be addressed to Lydie Izakovicova Holla; holla@med.muni.cz

Received 12 October 2018; Accepted 19 December 2018; Published 14 January 2019

Guest Editor: Maha El Tantawi

Copyright © 2019 Petra Borilova Linhartova et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Recent studies have suggested a bidirectional relationship between chronic periodontitis (CP) and diabetes mellitus (DM). Immunoregulatory factors such as cytokines play an important role in etiopathogenesis of both diseases. The aim of this study was to analyze variability in interleukin-1 (*IL-1*) gene cluster and IL-1β plasma levels in patients with CP, DM, and a combination of both diseases. A total of 1016 individuals participating in this case-control study—225 healthy controls, 264 patients with CP, 132 with type 1 diabetes (T1DM), and 395 patients with type 2 diabetes (T2DM)—were genotyped using methods based on polymerase chain reaction for *IL-1* gene polymorphisms (*IL-1A* (−889C/T, rs1800587), *IL-1B* (+3953C/T, rs1143634), and *IL-1RN* (gene for IL-1 receptor antagonist, IL-1RA, 86 bp tandem repeats in intron 2)). Levels of IL-1β were measured by Luminex methods in subgroups of controls, CP, T1DM + CP, and T2DM + CP subjects. Although no significant associations were found in the genotype and allele frequencies of *IL-1A* (−889C/T), significant differences in the allele frequencies of *IL-1B* (+3953C/T) were observed between controls and CP patients ($P < 0.05$). In T1DM patients, *IL-1RN*'S "short" allele and *IL-1RN* 12 genotype were significantly less frequent than those in controls ($P < 0.01$). In haplotype analysis, TTL haplotype decreased the risk of CP development ($P < 0.01$), whereas CCS and CTL haplotypes ($P < 0.01$ and $P < 0.05$) were associated with T1DM. Although IL-1β levels were measured significantly higher in mononuclear cells after stimulation by mitogens, HSP70, or selected periodontal bacteria than in unstimulated cells, *IL-1* genotypes did not correlate with circulating IL-1β levels. In the Czech population, significant associations between the *IL-1B* polymorphism with CP and the *IL-1RN* variant with T1DM were found. Haplotype analysis suggests that variability in *IL-1* gene cluster may be one of the factors in the CP and T1DM pathogenesis, although single variants of these polymorphisms are not substantial for protein production.

1. Introduction

Chronic periodontitis (CP), an inflammatory disease, which is the result of a complex interplay of bacterial infection and host responses, is characterized by the loss of connective tissue attachment, slowly progressing alveolar bone destruction, and, ultimately, loss of teeth. Molecular patterns (PAMPs) of anaerobic Gram-negative bacteria in the subgingival biofilm, among others, such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Tannerella forsythia* [1], are recognized by phagocytes. Macrophages produce proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), thus enhancing an immune response [2]. Elevation of these signal molecules in plasma/serum may lead to alterations in lipid metabolism and cause hyperlipidemia

[3]. In addition, in patients with diabetes mellitus (DM), periodontitis also adversely affects glycemic control [4].

DM results from insufficient insulin action (either absolute or relative). This most common metabolic disease is characterized by various degrees of chronic hyperglycemia, which together with an increased availability of free fatty acids is responsible for glucotoxicity and lipotoxicity in diabetes [5]. Apart from the systemic effects of diabetes, recent evidence suggests that local changes in the periodontal tissues are characterized by enhanced interactions between leukocytes and endothelial cells and altered leukocyte functions [6]. Increased levels of reactive oxygen species [7] and proinflammatory cytokines (IL-1β, IL-6, and TNF-α) lead to chronic low-grade inflammation, change of cytokine spectrum, and activation of innate immunity; alterations are present in both type 1 and type 2 diabetes mellitus (T1DM and T2DM, respectively) [8]. A meta-analysis by Chávarry et al. [9] identified T2DM as a moderate risk factor for CP, the effect of T1DM being less clear. It is probable that there is individual patient variability in the degree to which glycemic control influences periodontal status [10], and vice versa, periodontitis can contribute to the development of other diabetic complications, such as nephropathy (DN), retinopathy (DR), and neuropathy (DPN) [4].

IL-1α and IL-1β are produced during inflammation and induce production of many other cytokines, amplifying their proinflammatory action. They must be tightly controlled by endogenous inhibitors, such as IL-1 receptor antagonist (IL-1RA) and soluble IL-1 receptors, to prevent an acute and chronic overproduction of proinflammatory cytokines [11]. Polymorphisms in the *IL-1* cluster genes are located on chromosome 2q12 [12] and are composed of three ligands (*IL-1A*, *IL-1B*, and *IL-1RN*). To this date, only a few studies have focused on variability in the *IL-1* genes in diabetic patients with CP [13–17].

The first aim of this study was to investigate two single-nucleotide polymorphisms (SNPs) and one VNTR polymorphism in the *IL-1* gene cluster (*IL-1A* (−889C/T, rs1800587), *IL-1B* (+3953C/T, rs1143634), and *IL-1RN* (gene for IL-1 receptor antagonist, IL-1RA, 86 bp tandem repeats in intron 2)) in CP patients, diabetic patients of both types (T1DM and T2DM), and healthy controls in the Czech population. The second objective was to compare the subset of diabetic patients with CP and generally healthy patients without/with CP or diabetic patients according to their *IL-1* haplotype profile. Finally, assuming higher circulating IL-1β levels in diabetic patients with CP than in nondiabetic patients with CP, we, as the third aim, analyzed plasma levels in these groups of patients and their correlation to polymorphisms in the *IL-1* gene cluster. In addition, we examined IL-1β levels in mononuclear cells of generally healthy subjects after stimulation by oral pathogens, mitogens, or heat shock protein 70 (HSP70).

2. Materials and Methods

The study was performed with the approval of the Committees for Ethics of the Faculty of Medicine, Masaryk University, Brno (No. 13/2013), and St. Anne's Faculty Hospital. Written informed consent was obtained from all participants in line with the Declaration of Helsinki before inclusion in the study.

2.1. Subjects and Clinical Examinations. The cross-sectional study comprised a total of 1016 individuals, including 527 unrelated Caucasian subjects from the South Moravia region of the Czech Republic, with DM duration of at least 3 years. The diagnosis of T1DM (132 patients) or T2DM (395 patients) was originally based on the presence of clinical symptoms (such as polyuria, polydipsia, and weight loss) and biochemical parameters (glycemia, ketoacidosis, and autoantibody status in T1DM) in the outpatient unit of the diabetology centers in Brno. All patients were receiving antidiabetic drugs or insulin therapy and other medicaments according to the presence of diabetic complications, such as DN, DR, and DPN, and other comorbidities, as described in our cohort previously [18]. Levels of glycemia, glycated hemoglobin (HbA1c), total cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL), body mass index (BMI), and further parameters were recorded.

The periodontal status was evaluated in subgroups of the diabetic patients (38 T1DM and 89 T2DM patients), 225 healthy controls and 264 CP subjects recruited from the patient pool of the Clinic of Stomatology, St. Anne's Faculty Hospital, Brno, in the period of 2013–2017. The diagnosis of periodontitis/nonperiodontitis was based on the detailed clinical examination, medical and dental history, tooth mobility, and radiographic assessment [19]; the exclusion criteria for the studied cohort were described in our previous study [20].

2.2. Genetic Analysis. Genomic DNA was isolated from peripheral blood by a standard protocol and archived in the DNA bank at the Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic. Polymorphisms *IL-1A* (−889C/T, rs1800587) and *IL-1B* (+3953C/T, rs1143634) were genotyped by polymerase chain reaction, with restriction analysis (PCR-RFLP) according to protocols published previously [21, 22]. The VNTR PCR method was used for the detection of 86 bp tandem repeats number in the second intron of the *IL-1RN* gene [23]. *IL-1RN* alleles were coded as *IL-1RN**1 (4 repeats, 412 bp), *IL-1RN**2 (2 repeats, 240 bp), *IL-1RN**3 (3 repeats, 326 bp), *IL-1RN**4 (5 repeats, 498 bp), *IL-1RN**5 (6 repeats, 584 bp), and *IL-1RN**6 allele (1 repeat, 154 bp). Details and modifications of PCR conditions were published in our previous study by Izakovicova Holla et al. [24].

2.3. Plasma Level Analysis. Levels of IL-1β in plasma were measured in 60 randomly selected subjects (20 CP, 20 T1DM + CP, and 20 T2DM + CP patients) at the Department of Biochemistry, Faculty of Medicine, Masaryk University, Brno. Plasma samples were prepared from venous blood, collected into a tube with EDTA (S-Monovette® 9 mL K3E,

Sarstedt, Germany) by centrifugation (465 g/10 minutes/4°C), and stored at −70°C within 30 minutes after collection. For the determination of cytokine concentrations, Cytokine Human 10-Plex Panel for Luminex® Platform (Invitrogen™, California, USA), Luminex 200™ analyzer with xPONENT 3.1 Software (Luminex Corporation, USA), and Milliplex™ Analyst v 3.4 Software (VigeneTech, USA) were used.

In addition, examination of IL-1β levels in unstimulated and stimulated cells of 60 generally healthy subjects was performed in the laboratory of the Institute of Clinical and Experimental Dental Medicine, General University Hospital, and First Faculty of Medicine, Charles University, Prague. Isolation, cultivation, and stimulation of cells by selected periodontal bacteria (*A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, and *T. forsythia*), mitogens, or HSP70 were described previously [25]. IL-1β levels were determined in mononuclear cells isolated from 20 ml of heparinized blood using Fluorokine® MAP Human MultiAnalyte Profiling Base Kit, Luminex® 100™ analyzer, and Luminex 100 IS™ Software (R&D Systems, USA).

2.4. Statistical Analysis. Statistical analysis was performed using the statistical package Statistica v. 13 (StatSoft Inc., USA). Standard descriptive statistics were applied in the analysis: absolute and relative frequencies for categorical variables, and mean with standard deviation (SD) or median with quartiles for quantitative variables. To compare independent groups, one-way analysis of variance (ANOVA) and the Kruskal–Wallis ANOVA were performed to compare continuous variables. For exclusion of outlier values (IL-1β plasma levels), Grubb's test was used ($P < 0.05$). The allele frequencies were calculated from the observed numbers of genotypes. The differences in the allele frequencies were tested by Fisher's exact test; the Hardy–Weinberg equilibrium (HWE) and genotype frequencies were calculated by the chi-square test (χ^2). To examine the linkage disequilibrium (LD) between polymorphisms, pairwise LD coefficients (D′) and haplotype frequencies were calculated using the SNP Analyzer 2 program (http://snp.istech.info/istech/board/login_form.jsp). The association was described by odds ratios (OR) with 95% confidence intervals (95%CI). Only the values of P less than 0.05 were considered as statistically significant.

3. Results

3.1. Clinical Data Analysis. The mean ages of the healthy controls and patient with CP did not differ significantly ($P > 0.05$); however, patients with T1DM were significantly younger ($P < 0.05$), and in contrast, patients with T2DM were significantly older than those in healthy controls ($P < 0.01$). There were no significant differences between the subjects with T1DM/T2DM and/or CP and the controls relating to the male/female ratio. The BMI values were the highest in the groups of T2DM and T2DM + CP, which differed statistically significantly from other groups ($P < 0.05$). All T1DM ($N = 38$) and T2DM ($N = 89$) patients who were examined at the Department of Periodontology were affected by periodontitis. The duration of DM in the patients with T1DM was approximately double than that of DM in T2DM patients. Slightly lower HbA1c levels in groups of patients with T1DM + CP and T2DM + CP compared with the group of all DM patients can be given by a higher interest of these patients in their health condition (only these patients accepted the offer to be examined by periodontist). The frequencies of diabetic complications (DN, DR, and DPN) in the whole diabetic cohort vs. the subgroup of T1DM or T2DM patients with CP are summarized in Table 1.

3.2. SNPs Analysis. The frequencies of *IL-1* genotypes were in HWE in the control subjects ($P > 0.05$, see Table 2). *IL-1RN**5 allele (6 repeats, 584 bp) was not detected in our sample. For calculation of allele frequencies and haplotype analysis, alleles with more than two repeat units in the *IL-1RN* gene were grouped together as *IL-1RN**L ("long" allele) and allele with 2 repeats was marked as *IL-1RN**S ("short" allele) [26]. As *IL-1RN**6 allele (with 1 repeat, 154 bp) found in one CP subject cannot be considered as typical "short" allele, this sample was excluded from both these analyses.

None association between *IL-1A* (−889C/T) SNP or a so-called "double genotype" (*IL-1A* (−889C/T)/*IL-1B* (+3953C/T)) and CP and/or DM was found, but the *IL-1B**T allele (+3953C/T) was less frequent in CP patients than in healthy controls (21.4% vs. 28.0%, $P < 0.05$, OR = 0.70, 95% CI = 0.52–0.94). Significant differences were found in the *IL-1RN**S allele frequencies and also in *IL-1RN**12 (vs. *IL-1RN**11) genotype frequencies between T1DM patients and healthy controls ($P < 0.01$, OR = 0.64, 95%CI = 0.44–0.92 and $P < 0.01$, OR = 0.46, 95%CI = 0.28–0.76). Comparison of the allele frequencies of *IL-1RN* in patients with T1DM vs. T2DM showed a significant difference between these groups ($P < 0.05$).

3.3. Haplotype Analysis. Combination of multiple SNPs sites showed eight *IL-1* haplotypes with frequency more than 1% (see Table 3). Pairwise linkage disequilibrium (LD) for all possible 2-way comparisons among 3 polymorphisms in the *IL-1* gene cluster in CP, T1DM, T2DM, and control groups was measured by Lewontin standardized disequilibrium coefficient (|D′|, data not presented).

The distribution of these haplotype frequencies between controls and patient groups showed significant differences. Haplotype T[*IL-1A*(−889C/T)]/T[*IL-1B* (+3953C/T)]/L[*IL-1RN*] was associated with lower risks for CP ($P < 0.01$, OR=0.64, 95% CI = 0.47–0.88), similar to haplotype CCS ($P < 0.01$, OR = 0.58, 95% CI = 0.39–0.87) with T1DM. On the contrary, CTL haplotype ($P < 0.05$, OR = 1.98, 95% CI = 1.01–3.87) was found in a higher frequency in T1DM patients vs. healthy subjects.

3.4. IL-1β Plasma Level Analysis. No significant differences in IL-1β plasma levels in diabetic patients and generally healthy subjects with similar periodontal conditions were found (see Figure 1). In twenty examined patients (nine CP,

Table 1: Demographic data of the studied subjects with CP, T1DM (and T1DM + CP subgroup), T2DM (and T2DM + CP subgroup), and healthy controls.

Characteristics	Controls (N = 225)	CP (N = 264)	T1DM (N = 132)	T1DM + CP (N = 38)	T2DM (N = 395)	T2DM + CP (N = 89)
Age (mean years ± SD)	53.9 ± 11.0	56.1 ± 8.2	45.8 ± 14.2*	49.4 ± 10.9	67.5 ± 10.6*	67.3 ± 10.0*
Gender (males/females)	114/111	122/142	68/64	16/22	200/195	45/44
Smoking (no/yes, %)	72.9/27.1	71.6/28.4	—	76.3/23.7	—	87.7/12.3
BMI (mean ± SD)	23.2 ± 4.6	26.8 ± 4.0	24.5 ± 6.1	25.3 ± 3.1	28.6 ± 10.0*	30.3 ± 6.2*
Duration of DM (mean years ± SD)	n.a.	n.a.	21.5 ± 9.8	24.0 ± 10.7	15.1 ± 8.8	11.0 ± 8.7
HbA1c (mmol/mol, mean ± SD)	n.a.	n.a.	77.1 ± 17.3	69.8 ± 11.8	72.6 ± 23.9	58.0 ± 15.6
DN (no/yes, %)	n.a.	n.a.	43.6/56.4	71.0/29.0	7.6/92.4	80.2/19.8
DR (no/yes, %)	n.a.	n.a.	24.7/75.3	40.6/59.4	52.9/47.1	92.4/7.6
DPN (no/yes, %)	n.a.	n.a.	36.7/63.3	47.1/52.9	49.3/50.7	84.4/15.6

CP = chronic periodontitis; N = number of subjects; SD = standard deviation; T1DM = type 1 diabetes mellitus; T2DM = type 2 diabetes mellitus; BMI = body mass index; DN = diabetic nephropathy; DPN = diabetic peripheral neuropathy; DR = diabetic retinopathy; HbA1c = glycated hemoglobin; — = unknown in diabetic patients without CP; n.a. = nonapplicable. *$P < 0.05$ in comparison to healthy controls (evaluated by the Kruskal–Wallis ANOVA test).

five T1DM + CP, and six T2DM + CP), IL-1β plasma levels were under the detection limit (<2.58 pg/mL); for these samples, a value of 2.57 pg/mL was assigned for the statistical analyses. IL-1β plasma levels in the whole studied set (N = 60) were independent of the *IL-1B* or *IL-1RN* genotypes distribution (see Table 4); this result was also confirmed by the analysis of IL-1β levels in peripheral blood mononuclear cells (PBMCs) in generally healthy population (data not shown). However, high significant differences were observed in IL-1β levels between unstimulated PBMCs and cells after stimulation by Pokeweed mitogen (PWM), PWM in costimulation by Concavalin A (Con A), selected periodontal bacteria (*A. actinomycetemcomitans*, *P. intermedia*, and *T. forsythia*) (all $P < 0.001$), or HSP70 ($P < 0.02$, see Table 5).

4. Discussion

Diabetes and periodontitis are complex diseases with a bidirectional relationship [27]. Despite long-established evidence that hyperglycemia in diabetes is associated with adverse periodontal outcomes, the mechanism between these two states is not fully understood yet [10, 28]. Chronic inflammation, a common feature in the pathogenesis of both CP and DM, is related to the accumulation of activated innate immune cells in tissues, which results in the release of inflammatory mediators, such as IL-1 family cytokines. In this case-control study, we analyzed variability in the *IL-1* gene cluster and IL-1β plasma levels in patients with CP with/without diabetes in comparison to healthy controls. Minor allele frequency (MAF) of *IL-1A* (−889C/T) found in our healthy controls was 31%, which is in line with the *IL-1A**T allele frequency according to NCBI database (28%) (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=1800587), whereas in healthy European subjects, higher MAF was reported (37% [29]; 56%) [30]. The MAF of *IL-1B* (+3953C/T, rs1143634) in the Czech population (28%) was similar as *IL-1B**T allele frequency in healthy European subjects (from 24% to 29%) [29–32], whereas the NCBI database provides MAF of only 13% (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=1143634). *IL-1RN**S allele in European population varies from 21% to 31% [33–36], which is consistent with that in our observation (27%).

4.1. Chronic Periodontitis. In the context of CP, variability in the *IL-1* gene cluster has been investigated many times with conflicting results. To our knowledge, no study has examined the relationship between specific allele combinations of these three *IL-1* gene variants (*IL-1A* (−889C/T), *IL-1B* (+3953C/T), and *IL-1RN* (VNTR)) and periodontal diseases. In our population, haplotype TTL seems to be protective against the development of CP and the *IL-1B**T allele is of the same importance. This observation supports our previously detected association between *IL-1B**C allele and a higher risk for CP [37]. Nevertheless, da Silva et al. [38] came to opposite conclusions in their meta-analysis including 54 studies in different populations, which associated T allele in Caucasian carriers with a 1.25-time higher risk of developing CP than C allele carriers. In addition, no association between *IL-1A* (−889C/T) polymorphism and CP in the Czech population was found; this is in contrast to a recent meta-analysis by da Silva et al. [39]. In line with a meta-analysis by Ding et al. [40], we found no differences in allele or genotype frequencies in *IL-1RN* variants between CP patients and healthy controls.

We suggest that these discrepancies can be caused not only by population differences in allele frequencies but also by an interaction effect of *IL-1* genes variants. According to Morris and Kaplan [41], haplotype-based analysis can be more useful than an analysis based on individual polymorphisms in complex multifactorial diseases, as confirmed by our results.

Screening of SNPs and genome-wide studies has yielded new genetic information without a definitive solution for the management of periodontal disease [42]. However, SNP variations are no longer sufficient for establishing a relationship with periodontal disease. From this reason, the analyses of differential gene expression, performed using high-throughput experimental methods, such as microarray analysis, should be used in the future research [43]. Thus, the

Table 2: *IL-1* genotype and allele frequencies in patients with CP, T1DM (and T1DM + CP subgroup), T2DM (and T2DM + CP subgroup), and healthy controls.

Genotypes alleles	Controls (N = 225) N (%)	CP (N = 264) N (%)	OR (95% CI)	T1DM (N = 132) N (%)	OR (95% CI)	T1DM + CP (N = 38) N (%)	OR (95% CI)	T2DM (N = 395) N (%)	OR (95% CI)	T2DM + CP (N = 89) N (%)	OR (95% CI)
IL-1A (−889C/T)											
CC	105 (46.7)	145 (54.9)	1.00	61 (46.2)	1.00	20 (52.6)	1.00	198(50.1)	1.00	46 (51.7)	1.00
CT	102 (45.3)	104 (39.4)	0.74 (0.51–1.07)	62 (47.0)	1.05 (0.67–1.63)	15 (39.5)	0.77 (0.37–1.59)	160 (40.5)	0.83 (0.59–1.17)	34 (38.2)	0.76 (0.45–1.28)
TT	18 (8.0)	15 (5.7)	0.70 (0.34–1.36)	9 (6.8)	0.86 (0.36–2.03)	3 (7.9)	0.88 (0.24–3.25)	37 (9.4)	1.09 (0.59–2.01)	9 (10.1)	1.14 (0.48–2.73)
C allele	312 (69.3)	394 (74.6)	1.00	184 (69.7)	1.00	55 (72,4)	1.00	556 (70.4)	1.00	126 (70.8)	1.00
T allele	138 (30.7)	134 (25.4)	0.77 (0.58–1.02)	80 (30.3)	0.98 (0.71–1.37)	21 (27.6)	0.86 (0.50–1.48)	234 (29.6)	0.95 (0.74–1.22)	52 (29.2)	0.93 (0.64–1.36)
IL-1B (+3953C/T)											
CC	115 (51.1)	164 (62.1)	1.00	73 (55.3)	1.00	18 (47.4)	1.00	220 (55.7)	1.00	50 (56.2)	1.00
CT	94 (41.8)	87 (33.0)	0.65(0.45–0.95)	48 (36.4)	0.80 (0.51–1.27)	17 (44.7)	1.16 (0.56–2.37)	143 (36.2)	0.80 (0.56–1.12)	30 (33.7)	0.73 (0.43–1.25)
TT	16 (7.1)	13 (4.9)	0.57 (0.26–1.23)	11 (8.3)	1.08 (0.48–2.46)	3 (7.9)	1.20 (0.32–4.53)	32 (8.1)	1.05 (0.55–1.98)	9 (10.1)	1.29 (0.54–3.12)
C allele	324 (72.0)	415 (78.6)	1.00	194 (73.5)	1.00	53 (69.7)	1.00	584 (73.8)	1.00	130 (73.0)	1.00
T allele	126 (28.0)	113 (21.4)*	0.70 (0.52–0.94)	70 (26.5)	0.93 (0.66–1.31)	23 (30.3)	1.12 (0.66–1.90)	206 (26.2)	0.91 (0.70–1.18)	48 (27.0)	0.95 (0.64–1.40)
IL-1RN (VNTR)[$]											
11	108 (48.0)	121 (45.8)	1.00	83 (62.9)	1.00	19 (50.0)	1.00	220 (55.7)	1.00	48 (53.9)	1.00
12	87 (38.7)	110 (41.7)	1.13 (0.77–1.65)	31 (23.5)*	0.46 (0.28–0.76)	11 (28.9)	0.72 (0.32–1.59)	129 (32.7)	0.73 (0.51–1.04)	25 (28.1)	0.65 (0.37–1.13)
13	8 (3.6)	1 (0.4)	0.11 (0.01–0.91)	1 (0.8)	0.16 (0.02–1.33)	1 (2.6)	0.71 (0.08–6.01)	3 (0.8)	0.18 (0.05–0.71)	1 (1.1)	0.28 (0.03–2.31)
14	3 (1.3)	8 (3.0)	2.38 (0.62–9.20)	4 (3.0)	1.73 (0.38–7.96)	1 (2.6)	1.89 (0.19–19.19)	9 (2.3)	1.47 (0.39–5.55	4 (4.5)	3.00 (0.65–13.93)
22	17 (7.6)	17 (6.4)	0.89 (0.43–1.83)	8 (6.1)	0.61 (0.25–1.49)	2 (5.3)	0.67 (0.14–3.13)	31 (7.8)	0.90 (0.47–1.69)	9 (10.1)	1.19 (0.50–2.86)
23	0	1 (0.4)	#	1 (0.8)	#	1 (2.6)	#	0	#	0	#
24	2 (0.9)	5 (1.9)	2.23 (0.42–11.74)	3 (2.3)	1.95 (0.32–11.95)	2 (5.3)	5.68 (0.75–42.84)	3 (0.8)	0.74 (0.12–4.47)	2 (2.2)	1.38 (0.19–10.04)
44	0	0	#	1 (0.8)	#	1 (2.6)	#	0	#	0	#
16	0	1 (0.4) [$$]	#	0	#	0	#	0	#	0	#
L allele	327 (72.7)	376 (71.5)	1.00	213 (80.7)	1.00	58 (76.3)	1.00	596 (75.4)	1.00	133 (74.7)	1.00
S allele	123 (27.3)	150 (28.5)	1.06 (0.80–1.40)	51 (19.3)*	0.64 (0.44–0.92)	18 (23.7)	0.83 (0.47–1.46)	194 (24.6)	0.87 (0.67–1.13)	45 (25.3)	0.90 (0.61–1.34)

CI = confidence interval; CP = chronic periodontitis; OR = odds ratio; T1DM = type 1 diabetes mellitus; T2DM = type 2 diabetes mellitus. * $P < 0.05$ in comparison to healthy controls (evaluated by Fisher's exact test, without correction for multiple comparisons). #Cannot be assessed because of the small number. $Alleles with more than two repeat units were grouped together as *IL-1RN** L ("long" allele) and 2 repeat units were marked as *IL-1RN**S ("short" allele). $$Sample with rare genotype *IL-1RN*16 was excluded from allele frequencies and haplotype analysis.

Table 3: Estimated frequencies (%) of *IL-1* haplotypes in patients with CP, T1DM (and T1DM + CP subgroup), T2DM (and T2DM + CP subgroup), and healthy controls.

IL-1A (−889C/T)	*IL-1B* (+3953C/T)	*IL-1RN* (VNTR)$	Controls (N = 225)	CP (N = 264)	T1DM (N = 132)	T1DM + CP (N = 38)	T2DM (N = 395)	T2DM + CP (N = 89)
C	C	L	43.14	48.66	47.47	46.60	46.14	49.37
C	C	S	21.64	21.50	14.03*	17.39	18.11	17.86
T	T	L	21.35	13.35*	15.62	18.03	17.42	19.06
T	C	L	4.99	5.83	9.40	3.31	6.77	3.65
C	T	L	3.19	3.65	8.20*	8.38	5.12	2.65
T	T	S	2.23	2.53	2.70	3.85	2.79	2.16
T	C	S	2.10	3.76	2.59	2.44	2.65	4.35
C	T	S	1.36	0.73	—	—	1.02	0.91

CP = chronic periodontitis; T1DM = type 1 diabetes mellitus; T2DM = type 2 diabetes mellitus. $For haplotype analysis, alleles with more than two repeat units were grouped together as *IL-1RN**L ("long" allele) and 2 repeat units were marked as *IL-1RN**S ("short" allele). Haplotypes are ordered according to decreasing haplotype frequency in the healthy control subjects. *$P < 0.05$ in comparison to healthy controls (without correction for multiple comparisons).

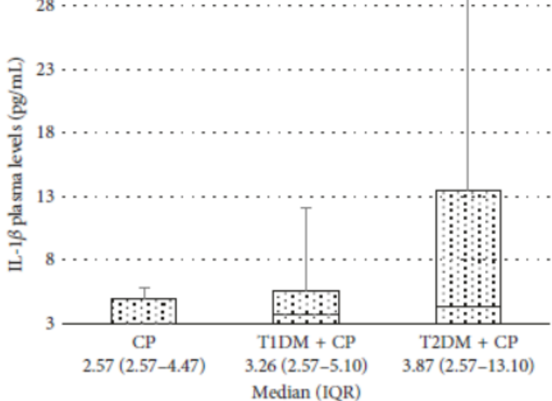


Figure 1: Comparison of IL-1β plasma levels in patients with CP (N = 20), T1DM + CP (N = 20), and T2DM + CP (N = 20). CP = chronic periodontitis, T1DM = type 1 diabetes mellitus, T2DM = type 2 diabetes mellitus. For samples with values of IL-1β under detection limit (<2.58 pg/mL) were assigned a value of 2.57 pg/mL. For exclusion of outlier values, three CP and one T1DM + CP samples were used in Grubb's test ($P < 0.05$). The Mann–Whitney U test (two-tail) was used to calculate significant differences.

Table 4: IL-1β plasma levels and polymorphisms in *IL-1B* and *IL-1RN* genes.

Genotypes	All groups (N = 60)	IL-1β levels (pg/mL), median (IQR)
IL-1B (+3953C/T)		
CC	31 (51.7)	3.26 (2.57–5.43)
CT	26 (43.3)	3.26 (2.57–10.59)
TT	3 (5.0)	2.69 (2.57–9.88)
IL-1RN (VNTR)$		
LL	37 (61.7)	3.26 (2.57–10.24)
LS	17 (28.3)	3.85 (2.57–9.35)
SS	5 (8.3)	3.26 (2.63–6.79)

IQR = interquartile range. $Alleles with more than two repeat units were grouped together as *IL-1RN**L ("long" allele) and 2 repeat units were marked as *IL-1RN**S ("short" allele); genotypes are known only in 59 subjects. The Kruskal–Wallis ANOVA test was used to calculate significant differences.

genetic basis of periodontal disease is moving from experimental evidence to a more consistent translation effect on diagnosis and development of new strategies to modulate the host [42].

4.2. T1DM. In our study, a significant association between "short" variant in intron 2 of the *IL-1RN* gene and lower risk of developing T1DM was observed ($P < 0.01$). Our findings are in line with the study in Egyptian population, where this minor allele and *IL-1RN* SS genotype were also less prevalent in patients with T1DM than in healthy controls [44]. On the other hand, in the recent study by Ali et al. [45], *IL-1RN**S allele and *IL-1RN* 12 genotype were present more frequently in the Saudi children with T1DM than healthy controls. *IL-1RN**S allele was previously associated with increased production of IL-1β [46] and IL-1RA, and also with reduced production of IL-1α by normal monocytes [47]. Thus, not only environmental factors as reported by Cullup et al. [48] but also variability in a number of 86 bp tandem repeats in intron 2 of the *IL-1RN* gene may be related to an imbalance between IL-1 anti- and proinflammatory protein levels.

In correlation to *IL-1* gene polymorphism, Krikovszky et al. [49] described the *IL-1B**T allele as risk in Hungarian children with T1DM. Although no significant relationships between *IL-1A* (−889C/T) or *IL-1B* (+3953C/T) variants and T1DM in Czech patients were found, the *IL-1B**T allele in combination with *IL-1A**C and *IL-1RN**L alleles was associated with an increased risk of T1DM.

Our results suggest that variability in the *IL-1* gene cluster, especially in the gene for IL-1RA, may be one of the factors in the pathogenesis of T1DM in Czech patients. This conclusion is supported by results from the haplotype analysis as the opposing haplotypes in *IL-1* CCS vs. CTL were significantly associated with lower vs. higher risk of T1DM.

As we supposed, variability in the *IL-1* gene cluster may play an important role in the pathogenesis of both diseases, and frequencies of the three most represented haplotypes (CCL, CCS, and TTL) in the T1DM + CP subjects seem to be intermediate between frequencies of CP and T1DM patients.

Table 5: Differences in IL-1β levels between unstimulated and stimulated PBMCs ($N = 60$).

PBMCs	IL-1β levels (pg/mL), median (IQR)	P value
Stimulated by		
PWM	481.97 (100.89–938.66)	<0.001
PWM + ConA	533.30 (141.45–950.37)	<0.001
HSP70	95.28 (23.36–371.60)	0.02
A. actinomycetemcomitans	483.93 (162.48–890.50)	<0.001
P. gingivalis	27.88 (6.47–118.14)	0.34
P. intermedia	249.38 (57.83–441.80)	<0.001
T. forsythia	201.08 (44.39–583.32)	<0.001
Unstimulated	52.55 (14.90–153.54)	—

CI = confidence interval; ConA = Concavalin A; HSP 70 = heat shock protein 70; OR = odds ratio; PBMCs = peripheral blood mononuclear cells; PWM = Pokeweed mitogen. The Wilcoxon matched pair test was used to calculate the significant difference.

Although a similar prevalence of CTL haplotype was found in the T1DM + CP subgroup and T1DM group, there was no statistical significance due to a low number of the examined type 1 diabetic patients with CP ($N = 38$).

4.3. T2DM. No significant differences in *IL-1A*, *IL-1B*, or *IL-1RN* gene variability between healthy controls and T2DM Czech patients with/without CP were found. Independently of periodontal status, Luotola et al. [50] suggested gender-specific associations between *IL-1A* (-899C/T) and *IL-1B* (+3953C/T) gene variants and a higher risk of T2DM, especially in men. Previously, the same authors described a relationship between *IL-1B**T allele (+3953C/T) and higher blood glucose levels in Finnish patients with DM [51]. In Malayalam-speaking Dravidian population, differences in *IL-1B**T allele and *IL-1B* TT genotype distribution between T2DM + CP ($N = 51$) and CP patients were found [16]. *IL-1RN**S allele and *IL-1RN* SS genotype were also associated with risk of developing T2DM in the Indian population [52, 53].

Our results correspond to observations in Chilean population focused on the same polymorphism in the *IL-1* gene cluster in T2DM and/or CP patients [15]. Deppe et al. [17] in their recent study assumed that CP in T2DM patients was most strongly associated with inadequate oral hygiene, whereas variability in the *IL-1* genes and differences in oral microbiota seemed to play a subordinate role. Furthermore, Guzman et al. [13] found only a borderline association between *IL-1B* (+3953C/T) SNP and the incidence of periodontal disease in Caribbean diabetic population. Struch et al. [14] reported that diabetic carriers of T allele in "double genotype" *IL-1A*/*IL-1B* had an enhanced risk for periodontal disease in comparison with their *IL-1* wild-type counterparts. Although the sample sizes in these three German studies ($N = 66$ [13] or 69 [14] or 38 patients with T2DM + CP [17]) are comparable with the size of our cohort ($N = 89$), Czech T2DM patients were selected from ethnically homogenous population, and in addition, other 306 T2DM patients with unknown periodontal status were included.

Results of haplotype analysis showed an interesting trend: the CCL, CCS, and TTL haplotype frequencies in the diabetic patients with CP were the highest or the lowest among CP, T2DM, and T2DM + CP patient groups. Although the TTL haplotype was considered protective for the development of CP in our population, frequency of this allele combination in T2DM + CP patients was closer to the results in healthy controls.

4.4. IL-1β Plasma Levels. IL-1β levels in gingival crevicular fluid (GCF) of patients with CP are significantly higher than those in patients with gingivitis and periodontally healthy individuals [54–56]. Therefore, elevated IL-1β GCF levels, but not plasma levels, were suggested as reliable inflammatory biomarkers in periodontal diseases [57]. In T1DM patients, increased levels of IL-1β in GCF were also found [58], and according to Aspriello et al. [59], IL-1β levels in T1DM patients with periodontitis are affected by the duration of DM. Meta-analysis by Atieh et al. [60] presented significant differences in IL-1β GCF levels between T2DM with CP patients and nondiabetic controls with the similar periodontal conditions.

Only a few studies reported inconsistent results for IL-1β concentrations in plasma/serum in relation to periodontal diseases or DM. Levels of circulating IL-1β were elevated in T1DM children than the control group [61, 62] and mRNA levels of IL-1β in peripheral blood leukocytes were found higher in T2DM patients than in healthy controls [63]. In another study, Sapathy et al. [64] suggested that changes of serum IL-1β levels were influenced by abdominal obesity and periodontal status independently even in the absence of DM and smoking.

In our study, the IL-1β plasma levels were under the detection limit in nine of 20 patients with CP. However, low values of this proinflammatory cytokine are not surprising because these patients are in good general health. Previously, Orozco et al. [54] also demonstrated zero concentration of IL-1β in the serum samples of both periodontitis and gingivitis patients. Nevertheless, Gümüş et al. [65] calculated median 11.7 pg/mL of serum IL-1β levels in their CP population. The higher circulating levels of IL-1β in 55% of Czech CP subjects may be the consequence of their smoking status since other risk factors linked with the increase in proinflammatory cytokines were minimized by strict criteria for inclusion in the study.

To our knowledge, this is the first study comparing IL-1β plasma levels in T2DM patients with CP and nondiabetic patients with CP. Although we assumed significant higher concentrations of this cytokine in all diabetic patients than in generally healthy patients with similar periodontal conditions, only a slightly elevated IL-1β levels were found in T1DM + CP or T2DM + CP patients compared with the CP subjects (median 3.26 pg/mL or 3.87 pg/mL vs. 2.57 pg/mL).

Furthermore, Santtila et al. [46] published that mononuclear cells from noncarriers of *IL-1B**T allele (+3953C/T) had a slight, but nonsignificantly, elevated capacity to produce IL-1β *in vitro*. Our results are in line with this finding; the single variants of *IL-1B* or *IL-1RN*

polymorphisms are not crucial in the protein production as apparent from both analyses of levels in plasma or in PBMCs. In our study, IL-1β levels in mononuclear cells of 60 generally healthy subjects after stimulation by periodontal bacteria (except *P. gingivalis*), mitogens, or HSP70 were examined. The obtained data confirmed our premise that these stressors significantly affect the production of proinflammatory IL-1β.

The main limitation of this study is the fact that only subgroups of diabetic patients with T1DM or T2DM were checked for their periodontal status as the number of patients willing to be examined at the Periodontology Department was low. On the other hand, the number of T2DM + CP patients is still higher than in previous studies in other populations. Besides the size of the overall study cohort ($N = 1016$), a further positive aspect of this study is a haplotype approach applied which presents a more complex view on the variation in the *IL-1* gene cluster. We assume that the discrepancy between our results and that from recent meta-analyses focusing on the relation between *IL-1* polymorphisms and CP can be partly caused by the fact that these meta-analyses studied only single variants in the *IL-1A* [38] or *IL-1B* genes [39] and by publication "bias" when negative results are not published.

5. Conclusions

In conclusion, results of the *IL-1* gene cluster analysis suggest that variability especially in the *IL-1B* and *IL-1RN* genes may be one of the factors in the susceptibility to T1DM and CP, although the single variants of these polymorphisms are not crucial for the protein production. In addition, no differences in IL-1β plasma levels were found between Czech diabetic patients with CP and generally healthy subjects with similar periodontal conditions.

Data Availability

The clinical and genetic data used to support the findings of this study are restricted by the Committees for Ethics of the Faculty of Medicine, Masaryk University, Brno (No. 13/2013), in order to protect patient privacy. Data are available from the corresponding author via mail for researchers who meet the criteria for access to confidential data.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

PBL was responsible for conceptualization, methodology, validation, formal analysis, investigation, data curation, visualization, and original draft preparation. HP, MT, JB, KK, and AF were responsible for methodology and reviewing and editing of the manuscript. LIH was responsible for conceptualization, methodology, software, validation, formal analysis, investigation, data curation, reviewing and editing of the manuscript, and supervision.

Acknowledgments

The authors would like to thank their students (laboratory technicians) Svetlana Lucanova and Jakub Kastovsky. This study was supported by the grant from the Czech Science Foundation (GB14-37368G), the project of Grant Agency of Masaryk University (MUNI/A/1008/2017), and funds provided by the Faculty of Medicine, MU, to the junior researcher Petra Borilova Linhartova.

References

[1] S. S. Socransky, A. D. Haffajee, M. A. Cugini, C. Smith, and R. L. Kent Jr, "Microbial complexes in subgingival plaque," *Journal of Clinical Periodontology*, vol. 25, no. 2, pp. 134–144, 1998.

[2] P. N. Madianos, Y. A. Bobetsis, and D. F. Kinane, "Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingiva," *Journal of Clinical Periodontology*, vol. 32, no. 6, pp. 57–71, 2005.

[3] A. M. Iacopino, "Periodontitis and diabetes interrelationships: role of inflammation," *Annals of Periodontology*, vol. 6, no. 1, pp. 125–137, 2001.

[4] E. Lalla and P. N. Papapanou, "Diabetes mellitus and periodontitis: a tale of two common interrelated diseases," *Nature Reviews Endocrinology*, vol. 7, no. 12, pp. 738–748, 2011.

[5] V. Poitout, J. Amyot, M. Semache, B. Zarrouki, D. Hagman, and G. Fontés, "Glucolipotoxicity of the pancreatic beta cell," *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, vol. 1801, no. 3, pp. 289–298, 2010.

[6] S. K. Sonnenschein and J. Meyle, "Local inflammatory reactions in patients with diabetes and periodontitis," *Periodontol 2000*, vol. 69, no. 1, pp. 221–254, 2015.

[7] L. J. Yan, "Pathogenesis of chronic hyperglycemia: from reductive stress to oxidative stress," *Journal of Diabetes Research*, vol. 2014, Article ID 137919, 11 pages, 2014.

[8] J. I. Odegaard and A. Chawla, "Connecting type 1 and type 2 diabetes through innate immunity," *Cold Spring Harbor Perspectives in Medicine*, vol. 2, no. 3, article a007724, 2012.

[9] N. G. Chávarry, M. V. Vettore, C. Sansone, and A. Sheiham, "The relationship between diabetes mellitus and destructive periodontal disease: a meta-analysis," *Oral Health and Preventive Dentistry*, vol. 7, no. 2, pp. 107–27, 2009.

[10] B. L. Mealey, "Periodontal disease and diabetes: a two-way street," *Journal of the American Dental Association*, vol. 137, pp. 26–31, 2006.

[11] C. A. Dinarello, "Interleukin-1 and interleukin-1 antagonism," *Blood*, vol. 77, pp. 1627–52, 1991.

[12] A. Steinkasserer, N. K. Spurr, S. Cox, P. Jeggo, and R. B. Sim, "The human IL-1 receptor antagonist gene (IL1RN) maps to chromosome 2q14-q21, in the region of the IL-1α and IL-1β loci," *Genomics*, vol. 13, pp. 654–657, 1992.

[13] S. Guzman, M. Karima, H.-Y. Wang, and T. E. V. Dyke, "Association between interleukin-1 genotype and periodontal disease in a diabetic population," *Journal of Periodontology*, vol. 74, no. 8, pp. 1183–1190, 2003.

[14] F. Struch, M. Dau, C. Schwahn, R. Biffar, T. Kocher, and P. Meisel, "Interleukin-1 gene polymorphism, diabetes, and periodontitis: results from the Study of Health in Pomerania (SHIP)," *Journal of Periodontology*, vol. 79, no. 3, pp. 501–507, 2008.

[15] N. J. López, C. Y. Valenzuela, and L. Jara, "Interleukin-1 gene cluster polymorphisms associated with periodontal disease in

type 2 diabetes," *Journal of Periodontology*, vol. 80, no. 10, pp. 1590–1598, 2009.

[16] N. Sharma, R. Joseph, R. Arun, R. Chandni, K. L. Srinivas, and M. Banerjee, "Cytokine gene polymorphism (interleukin-1β +3954, interleukin-6 [-597/-174] and tumor necrosis factor-α -308) in chronic periodontitis with and without type 2 diabetes mellitus," *Indian Journal of Dental Research*, vol. 25, no. 3, pp. 375–380, 2014.

[17] H. Deppe, T. Mücke, S. Wagenpfeil et al., "Are selected IL-1 polymorphisms and selected subgingival microorganisms significantly associated to periodontitis in type 2 diabetes patients? a clinical study," *BMC Oral Health*, vol. 15, no. 1, p. 143, 2015.

[18] L. Pácal, J. Varvařovská, Z. Rušavý et al., "Parameters of oxidative stress, DNA damage and DNA repair in type 1 and type 2 diabetes mellitus," *Archives of Physiology and Biochemistry*, vol. 117, no. 4, pp. 222–230, 2011.

[19] P. Borilova Linhartova, J. Vokurka, H. Poskerova, A. Fassmann, and L. Izakovicova Holla, "Haplotype analysis of interleukin-8 gene polymorphisms in chronic and aggressive periodontitis," *Mediators of Inflammation*, vol. 2013, p. 342351, 2013.

[20] P. Borilova Linhartova, J. Kastovsky, S. Lucanova et al., "Interleukin-17A gene variability in patients with type 1 diabetes mellitus and chronic periodontitis: its correlation with IL-17 levels and the occurrence of periodontopathic bacteria," *Mediators of Inflammation*, vol. 2016, Article ID 2979846, 9 pages, 2016.

[21] T. L. Mcdowell, J. A. Symons, R. Ploski, Ø. FØRre, and G. W. Duff, "A genetic association between juvenile rheumatoid arthritis and a novel interleukin-1α polymorphism," *Arthritis and Rheumatism*, vol. 38, no. 2, pp. 221–228, 1995.

[22] K. S. Kornman, A. Crane, H.-Y. Wang et al., "The interleukin-1 genotype as a severity factor in adult periodontal disease," *Journal of Clinical Periodontology*, vol. 24, no. 1, pp. 72–77, 1997.

[23] J. K. Tarlow, A. I. Blakemore, A. Lennard et al., "Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat," *Human Genetics*, vol. 91, no. 4, pp. 403-404, 1993.

[24] L. Izakovicova Holla, P. Borilova Linhartova, B. Hrdlickova et al., "Haplotypes of the IL-1 gene cluster are associated with gastroesophageal reflux disease and Barrett's esophagus," *Human Immunology*, vol. 74, no. 9, pp. 1161–1169, 2013.

[25] J. Bartova, P. Borilova Linhartova, S. Podzimek et al., "The effect of IL-4 gene polymorphisms on cytokine production in patients with chronic periodontitis and in healthy controls," *Mediators Inflamm*, vol. 2014, article 185757, 11 pages, 2014.

[26] B. Tolusso, D. Pietrapertosa, A. Morelli et al., "IL-1B and IL-1RN gene polymorphisms in rheumatoid arthritis: relationship with protein plasma levels and response to therapy," *Pharmacogenomics*, vol. 7, pp. 683–695, 2006.

[27] P. M. Preshaw, A. L. Alba, D. Herrera et al., "Periodontitis and diabetes: a two-way relationship," *Diabetologia*, vol. 55, no. 1, pp. 21–31, 2011.

[28] A. Bascones-Martínez, J. González-Febles, and J. Sanz-Esporrín, "Diabetes and periodontal disease. Review of the literature," *American Journal of Dentistry*, vol. 27, no. 2, pp. 63–67, 2014.

[29] P. M. Brett, P. Zygogianni, G. S. Griffiths et al., "Functional gene polymorphisms in aggressive and chronic periodontitis," *Journal of Dental Research*, vol. 84, no. 12, pp. 1149–1153, 2016.

[30] J. Wagner, W. E. Kaminski, C. Aslanidis et al., "Prevalence of OPG and IL-1 gene polymorphisms in chronic periodontitis," *Journal of Clinical Periodontology*, vol. 34, no. 10, pp. 823–827, 2007.

[31] D. Sakellari, S. Koukoudetsos, M. Arsenakis, and A. Konstantinidis, "Prevalence of IL-1A and IL-1B polymorphisms in a Greek population," *Journal of Clinical Periodontology*, vol. 30, no. 1, pp. 35–41, 2003.

[32] A. Bascones-Martínez, G. Valderrama, F. Vijande et al., "Interleukin-1 gene polymorphisms and periodontal status in a Spanish population," *Molecular Medicine Reports*, vol. 5, no. 5, pp. 1335–1339, 2012.

[33] J. M. Parkhill, B. J. W. Hennig, I. L. C. Chapple, P. A. Heasman, and J. J. Taylor, "Association of interleukin-1 gene polymorphisms with early-onset periodontitis," *Journal of Clinical Periodontology*, vol. 27, no. 9, pp. 682–689, 2000.

[34] M. L. Laine, M. A. Farre, M. A. Garcia-González et al., "Polymorphisms of the interleukin-1 gene family, oral microbial pathogens, and smoking in adult periodontitis," *Journal of Dental Research*, vol. 80, no. 8, pp. 1695–1699, 2016.

[35] D. Sakellari, V. Katsares, M. Georgiadou, A. Kouvatsi, M. Arsenakis, and A. Konstantinidis, "No correlation of five gene polymorphisms with periodontal conditions in a Greek population," *Journal of Clinical Periodontology*, vol. 33, no. 11, pp. 765–770, 2006.

[36] A. Havemose-Poulsen, L. K. Sørensen, K. Bendtzen, and P. Holmstrup, "Polymorphisms within the IL-1 gene cluster: effects on cytokine profiles in peripheral blood and whole blood cell cultures of patients with aggressive periodontitis, juvenile idiopathic arthritis, and rheumatoid arthritis," *Journal of Periodontology*, vol. 78, no. 3, pp. 475–492, 2007.

[37] L. Izakovičová Hollá, N. Dvořáková, T. Halaba, V. Znojil, J. Vácha, and J. Vaněk, "Analýza polymorfismů v genech pro interleukin-1 (IL-1) u českých pacientů s chronickou parodontitidou," *Česká stomatologie*, vol. 104, no. 2, pp. 48–56, 2004.

[38] F. R. P. da Silva, A. C. C. G. Vasconcelos, L. F. de Carvalho França, D. Di Lenardo, H. M. S. Nascimento, and D. F. P. Vasconcelos, "Association between the rs1143634 polymorphism in interleukin-1B and chronic periodontitis: results from a meta-analysis composed by 54 case/control studies," *Gene*, vol. 668, pp. 97–106, 2018.

[39] F. R. da Silva, A. C. Guimarães-Vasconcelos, L. F. de-Carvalho-França et al., "Relationship between -889 C/T polymorphism in interleukin-1A gene and risk of chronic periodontitis: evidence from a meta-analysis with new published findings," *Medicina Oral Patología Oral y Cirugia Bucal*, vol. 22, no. 1, pp. e7–e14, 2017.

[40] C. Ding, L. Zhao, Y. Sun, L. Li, and Y. Xu, "Interleukin-1 receptor antagonist polymorphism (rs2234663) and periodontitis susceptibility: a meta-analysis," *Archives of Oral Biology*, vol. 57, no. 6, pp. 585–593, 2012.

[41] R. W. Morris and N. L. Kaplan, "On the advantage of haplotype analysis in the presence of multiple disease susceptibility alleles," *Genetic Epidemiology*, vol. 23, no. 3, pp. 221–233, 2002.

[42] M. Taba Jr, S. L. Souza, and V. C. Mariguela, "Periodontal disease: a genetic perspective," *Brazilian Oral Research*, vol. 26, no. 1, pp. 32–38, 2012.

[43] X. Guo, Y. Wang, C. Wang, and J. Chen, "Identification of several hub-genes associated with periodontitis using integrated microarray analysis," *Molecular Medicine Reports*, vol. 11, no. 4, pp. 2541–2547, 2014.

[44] A. Settin, A. Ismail, M. A. El-Magd, R. El-Baz, and A. Kazamel, "Gene polymorphisms of TNF-α–308(G/A), IL-10–1082(G/A), IL-6–174(G/C) and IL-1Ra (VNTR) in Egyptian cases with type 1 diabetes mellitus," *Autoimmunity*, vol. 42, no. 1, pp. 50–55, 2009.

[45] R. A. Ali, L. M. Saber, and A. M. Al-Harbi, "A novel association between IL1-Ra (receptor antagonist) gene polymorphism and T1DM in Al-Madina Al-Mounawra," *European Review for Medical and Pharmacological Sciences*, vol. 19, no. 19, pp. 3701–3708, 2015.

[46] S. Santtila, K. Savinainen, and M. Hurme, "Presence of the IL-1RA allele 2 (IL1RN 2) is associated with enhanced IL-1beta production in vitro," *Scandinavian Journal of Immunology*, vol. 47, no. 3, pp. 195–198, 1998.

[47] V. A. Danis, M. Millington, V. J. Hyland, and D. Grennan, "Cytokine production by normal human monocytes: inter-subject variation and relationship to an IL-1 receptor antagonist (IL-1Ra) gene polymorphism," *Clinical and Experimental Immunology*, vol. 99, no. 2, pp. 303–310, 1995.

[48] H. Cullup, P. G. Middleton, G. Duggan, J. S. Conn, and A. M. Dickinson, "Environmental factors and not genotype influence the plasma level of interleukin-1 receptor antagonist in normal individuals," *Clinical and Experimental Immunology*, vol. 137, no. 2, pp. 351–358, 2004.

[49] D. Krikovszky, B. Vásárhelyi, A. Treszl et al., "Genetic polymorphism of interleukin-1β is associated with risk of type 1 diabetes mellitus in children," *European Journal of Pediatrics*, vol. 161, no. 9, pp. 507-508, 2002.

[50] K. Luotola, A. Pietilä, T Zeller et al., "Association between interleukin-1 (IL-1) gene variations of IL-1 receptor antagonist and the development type 2 diabetes," *Journal of Internal Medicine*, vol. 269, no. 3, pp. 322–332, 2011.

[51] K. Luotola, R. Pääkkönen, M. Alanne et al., "Association of variation in the interleukin-1 gene family with diabetes and glucose homeostasis," *J Clin Endocrinol Metab*, vol. 94, no. 11, pp. 4575–4583, 2009.

[52] B. R. Achyut, A. Srivastava, S. Bhattacharya, and B. Mittal, "Genetic association of interleukin-1β (−511C/T) and interleukin-1 receptor antagonist (86 bp repeat) polymorphisms with Type 2 diabetes mellitus in North Indians," *Clinica Chimica Acta*, vol. 377, no. 1-2, pp. 163–169, 2007.

[53] M. Banerjee, H. Bid, R. Konwar, and CG. Agrawal, "Association of IL-4 and IL-1RN (receptor antagonist) gene variants and the risk of type 2 diabetes mellitus: a study in the north Indian population," *Indian Journal of Medical Sciences*, vol. 62, no. 7, pp. 259–266, 2008.

[54] A. Orozco, E. Gemmell, M. Bickel, and G. J. Seymour, "Interleukin-1 beta, interleukin-12 and interleukin-18 levels in gingival fluid and serum of patients with gingivitis and periodontitis," *Oral Microbiology and Immunology*, vol. 21, no. 4, pp. 256–260, 2006.

[55] Y. Zhong, G. D. Slade, J. D. Beck, and S. Offenbacher, "Gingival crevicular fluid interleukin-1?, prostaglandin E2and periodontal status in a community population," *Journal of Clinical Periodontology*, vol. 34, no. 4, pp. 285–293, 2007.

[56] A. S. Ertugrul, H. Sahin, A. Dikilitas, N. Alpaslan, and A. Bozoglan, "Comparison of CCL28, interleukin-8, interleukin-1β and tumor necrosis factor-alpha in subjects with gingivitis, chronic periodontitis and generalized aggressive periodontitis," *Journal of Periodontal Research*, vol. 48, no. 1, pp. 44–51, 2012.

[57] S. Becerik, V. Ö. Öztürk, H. Atmaca, G. Atilla, and G. Emingil, "Gingival crevicular fluid and plasma acute-phase cytokine levels in different periodontal diseases," *Journal of Periodontology*, vol. 83, no. 10, pp. 1304–1313, 2012.

[58] G. E. Salvi, J. D. Beck, and S. Offenbacher, "PGE2, IL-1 β, and TNF-α responses in diabetics as modifiers of periodontal disease expression," *Annals of Periodontology*, vol. 3, no. 1, pp. 40–50, 1998.

[59] S. D. Aspriello, A. Zizzi, G. Tirabassi et al., "Diabetes mellitus-associated periodontitis: differences between type 1 and type 2 diabetes mellitus," *Journal of Periodontal Research*, vol. 46, no. 2, pp. 164–169, 2010.

[60] M. A. Atieh, C. M. Faggion Jr, and G. J. Seymour, "Cytokines in patients with type 2 diabetes and chronic periodontitis: a systematic review and meta-analysis," *Diabetes Research and Clinical Practice*, vol. 104, no. 2, pp. e38–e45, 2014.

[61] F. Pérez, A. Oyarzún, E. Carrasco, B. Angel, C. Albala, and J. L. Santos, "Plasma levels of interleukin-1beta, interleukin-2 and interleukin-4 in recently diagnosed type 1 diabetic children and their association with beta-pancreatic autoantibodies," *Revista Mèdica de Chile*, vol. 132, no. 4, pp. 413–420, 2004.

[62] K. Alnek, K. Kisand, K. Heilman, A. Peet, K. Varik, and R. Uibo, "Increased blood levels of growth factors, proinflammatory cytokines, and Th17 cytokines in patients with newly diagnosed type 1 diabetes," *PLoS One*, vol. 10, no. 12, Article ID e0142976, 2015.

[63] F. Liu, Y. Fu, C. Wei, Y. Chen, S. Ma, and W. Xu, "The expression of GPR109A, NF-kB and IL-1β in peripheral blood leukocytes from patients with type 2 diabetes," *Annals of Clinical and Laboratory Science*, vol. 44, no. 4, pp. 443–448, 2014.

[64] A. Satpathy, S. Ravindra, S. Thakur, S. Kulkarni, A. Porwal, and S. Panda, "Serum interleukin-1β in subjects with abdominal obesity and periodontitis," *Obesity Research and Clinical Practice*, vol. 9, no. 5, pp. 513–521, 2015.

[65] P. Gümüş, N. Nizam, A. Nalbantsoy, Ö Özçaka, and N. Buduneli, "Saliva and serum levels of pentraxin-3 and interleukin-1β in generalized aggressive or chronic periodontitis," *Journal of Periodontology*, vol. 85, no. 3, pp. e40–46, 2014.

4.2 Comment to the article ”Differences in interleukin-8 plasma levels between diabetic patients and healthy individuals independently on their periodontal status”

Borilova Linhartova P, Kavrikova D, Tomandlova M, Poskerova H, Rehka V, Dušek L, Izakovicova Holla L. Differences in interleukin-8 plasma levels between diabetic patients and healthy individuals independently on their periodontal status. Int J Mol Sci. 2018;19(10), pii: E3214. DOI: 10.3390/ijms19103214. (IF 3.687; BIOCHEMISTRY & MOLECULAR BIOLOGY Q2, CHEMISTRY, MULTIDISCIPLINARY Q2)

Author contribution: P.B.L. designed the study, drafted the paper, and carried out the genetic analysis.

Although no association had previously been found between *IL-8* gene polymorphisms and periodontal diseases in the Czech population, some *IL-8* variants have been associated with the presence of some periodontal bacteria as well as specific *IL-8* haplotypes were suggested to be protective against CP development (Bořilová Linhartová et al., 2013). Lappin et al. (2015) suggested that elevated IL-8 plasma levels potentially contribute to the cross-susceptibility between periodontitis and T1DM, and that *P. gingivalis* lipopolysaccharides (LPS) and advanced glycation end products (AGE) in combination caused significantly greater expression of IL-6, IL-8, and CXCL5 from THP-1 monocytes and OKF6/TERT-2 cells than LPS alone. In addition, chemokine receptor 2 (CXCR2) plays an important role in the regulation of the inflammatory response.

Considering the critical function of chemokine IL-8 in the inflammatory process, the aims of this study were to determine: (i) IL-8 plasma levels; (ii) *IL-8* rs4073 and its receptor 2 (*CXCR2*) rs1126579 polymorphisms, and (iii) the presence of the selected periodontal bacteria in T1DM and T2DM patients and the HC with known periodontal status.

This case-control study is comprised of 153 unrelated individuals: 36/44 patients suffering from T1DM+CP/T2DM+CP and 32/41 from the HC+CP/non-periodontitis HC. Both the clinical and biochemical parameters were monitored. The genotypes were determined using TaqMan PCR, IL-8 plasma levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit. Subgingival bacterial colonization was analyzed with a DNA microarray detection kit in an external laboratory (Stomagene, České Budějovice, Czech Republic).

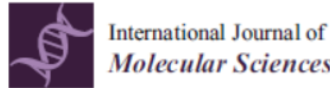
IL-8 rs4073 and *CXCR2* rs1126579 allelic and genotype distributions were similar in all of the subgroups and the T2DM+CP group between well/satisfactorily controlled individuals vs. unsatisfactorily controlled patients ($P>0.05$). Variants in both genes were also evaluated

across the whole cohort in relation to IL-8 plasma levels and no association was found ($P>0.05$).

The IL-8 plasma levels differed significantly between the non-periodontitis HC and T1DM+CP/T2DM+CP patients ($P<0.01$). Even in the HC+CP, IL-8 concentrations were significantly lower than in T1DM+CP/T2DM+CP patients ($P<0.05$).

Higher IL-8 plasma levels were found in the healthy controls and the well/satisfactorily controlled T2DM+CP patients who tested positive for *T. forsythia* or *P. intermedia* than in individuals from this subgroup who tested negative for these specific bacteria ($P=0.03$ in both, $P_{corr}>0.05$). However, the presence of *P. gingivalis*, *T. forsythia*, or *T. denticola* was associated with lower IL-8 plasma levels in T1DM+CP patients ($P<0.01$, $P_{corr}>0.05$). *F. nucleatum* was detected in almost all of the individuals (98.6%). IL-8 levels were found to be higher in the absence of *F. nucleatum* than in the presence of this bacterium in HC+CP individuals (mean 46.40 $pgmL^{-1}$ vs. 10.80 $pgmL^{-1}$), and in unsatisfactorily controlled T2DM+CP patients (mean 30.86 $pgmL^{-1}$ vs. 13.77 $pgmL^{-1}$). However, the significance of these results is questionable due to the low number of patients who tested negative for *F. nucleatum*.

In conclusion, no significant associations between IL-8 plasma levels and the studied *IL-8* and *CXCR2* polymorphisms or the occurrence of selected periodontal bacteria ($P_{corr}> 0.05$) were found. Patients with T1DM+CP/T2DM+CP had higher circulating IL-8 levels than the HC+CP/non-periodontitis HC.



Article

Differences in Interleukin-8 Plasma Levels between Diabetic Patients and Healthy Individuals Independently on Their Periodontal Status

Petra Borilova Linhartova [1,2,†], Denisa Kavrikova [1,†], Marie Tomandlova [3], Hana Poskerova [1], Vaclav Rehka [4], Ladislav Dušek [5] and Lydie Izakovicova Holla [1,2,*]

1. Clinic of Stomatology, Institution Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Pekarska 664/53, 60200 Brno, Czech Republic; peta.linhartova@gmail.com (P.B.L.); denisakavrikova@gmail.com (D.K.); hana.poskerova@fnusa.cz (H.P.)
2. Department of Pathophysiology, Faculty of Medicine, Masaryk University, Kamenice 5, 62500 Brno, Czech Republic
3. Department of Biochemistry, Faculty of Medicine, Masaryk University, Kamenice 5, 62500 Brno, Czech Republic; mhyks@med.muni.cz
4. Faculty of Medicine, Masaryk University, Kamenice 5, 62500 Brno, Czech Republic; vasek.rehka@seznam.cz
5. Institute of Biostatistics and Analyses, Faculty of Medicine, Masaryk University, 62500 Brno, Czech Republic; dusek@iba.muni.cz

* Correspondence: holla@med.muni.cz; Tel.: +420-543-183-406

† Authors contributed equally to this work.

Received: 29 September 2018; Accepted: 15 October 2018; Published: 18 October 2018

Abstract: Chronic periodontitis (CP) and diabetes mellitus (DM) involve several aspects of immune functions, including neutrophil activity and cytokine biology. Considering the critical function of chemokine interleukin-8 (IL-8) in the inflammatory process, the aims of this study were to determine: (i) IL-8 plasma levels; (ii) *IL-8* (−251A/T, rs4073) and its receptor 2 (*CXCR2*, +1208C/T, rs1126579) polymorphisms, and (iii) the presence of the selected periodontal bacteria in types 1 and 2 DM patients (T1DM and T2DM) and systemically healthy controls (HC) with known periodontal status. This case–control study comprises of 153 unrelated individuals: 36/44 patients suffering from T1DM+CP/T2DM+CP and 32/41 from HC+CP/non-periodontitis HC. Both the clinical and biochemical parameters were monitored. The genotypes were determined using qPCR, IL-8 plasma levels were measured using an ELISA kit. Subgingival bacterial colonization was analyzed with a DNA microarray detection kit. The IL-8 plasma levels differed significantly between non-periodontitis HC and T1DM+CP/T2DM+CP patients ($P < 0.01$). Even in HC+CP, IL-8 concentrations were significantly lower than in T1DM+CP/T2DM+CP patients ($P \leq 0.05$). No significant associations between the IL-8 plasma levels and the studied *IL-8* and *CXCR2* polymorphisms or the occurrence of selected periodontal bacteria ($P > 0.05$) were found. CP does not influence the circulating IL-8 levels. Patients with T1DM+CP/T2DM+CP had higher circulating IL-8 levels than HC+CP/non-periodontitis HC.

Keywords: interleukin-8; chemokines; plasma; polymorphism; chronic periodontitis; diabetes mellitus

1. Introduction

Diabetes mellitus (DM), most frequently occurring as type 1 (T1DM) and type 2 (T2DM), is a chronic metabolic disorder, which impacts global health [1]. The major cause of morbidity and early mortality in diabetic patients stems from vascular complications [2], developing as a consequence of long-term hyperglycemia and the formation of advanced glycation end products (AGE) [3]. The five

main complications of DM include retinopathy (DR), neuropathy (DPN), nephropathy (DN), altered wound healing, and macrovascular disease [4]. Chronic periodontitis (CP) is considered the sixth complication of diabetes [5], and it may have an increased influence on systemic levels of cytokines, especially in individuals with T2DM [6].

CP is mainly caused by Gram-negative bacteria in the subgingival biofilm, such as *Porphyromonas gingivalis* and *Tannerella forsythia* [7,8], and by "genetic dysbiosis," which highlights the role of human genetic variants affecting microbial recognition and host response in creating an environment conductive to changes in the normal microbiota [9]. A common feature in DM and CP patients is a low-grade inflammatory state [10,11], which suggests a link between the two diseases. DM has many adverse effects on the periodontium, including an impaired neutrophil function and the production of cytokines [12].

Interleukin-8 (IL-8), a member of the C-X-C motif (CXC) subfamily of chemokines, is one of the most important chemoattractants and activators of human neutrophils via interaction with two receptors (CXCR1 and CXCR2) [13]. IL-8 is involved in the initiation and amplification of acute inflammatory reaction; it is secreted by several cell types in response to inflammatory stimuli [14]. Chemokines and neutrophils have previously been associated with, or implicated in the pathogenesis of T1DM [15–18]. Furthermore, the study of non-obese diabetic mouse models has identified CXCR1/2 chemokine receptors as "master regulators" of diabetes pathogenesis [19,20]. Nevertheless, the role of IL-8 in DM and CP pathogenesis remains unclear.

The levels of IL-8 in oral keratinocytes [21], gingival epithelial cells [22], gingival crevicular fluid (GCF) [23–25], plasma [21], or serum [26] have been observed with contradictory findings in the context of DM and CP. It is highly probable that *IL-8* (−251A/T, rs4073) polymorphism affects the ability of individuals to produce IL-8 [27]. Furthermore, expression levels of *CXCR2* or specific *CXCR2* gene variants (+1208C/T, rs1126579) have been linked with levels of circulating IL-8 [28,29]. Although no association was previously found between the *IL-8* gene polymorphisms and periodontal diseases in the Czech population, *IL-8* variants have been associated with the presence of some periodontal bacteria as well as specific *IL-8* haplotypes were suggested to be protective against CP development [30].

Considering the critical function of IL-8 in inflammation, and its possible role in the pathogenesis of DM and CP, the aims of this study were to determine (i) IL-8 plasma levels; (ii) *IL-8* (−251A/T, rs4073) and its receptor 2 (*CXCR2*, +1208C/T, rs1126579) polymorphisms; and (iii) the presence of the selected periodontal bacteria in types 1 and 2 DM patients (T1DM and T2DM) and systemically healthy controls (HC) with known periodontal status.

2. Results

2.1. Study Population

The demographic data for the study population are shown in Table 1. The subgroups did not differ in terms of the male/female ratio ($P > 0.05$). The mean ages and BMI were similar for patients with T1DM+CP and HC, but there were significant differences in mean ages between groups of HC+CP/T2DM+CP and non-periodontitis HC (mean ± standard deviation, SD: 59.5 ± 9.3/66.8 ± 8.5 vs. 45.5 ± 9.6, $P < 0.01$). Mean body mass index (BMI) was significantly higher in T2DM+CP patients than in non-periodontitis HC and T1DM+CP patients (29.9 ± 7.7 vs. 23.8 ± 4.2/25.1 ± 3.1, $P < 0.05$). Smoking status was not different between T2DM+CP and non-periodontitis HC (5.3% vs. 7.1% smokers) or between T1DM+CP patients and HC+CP individuals (19.4% vs. 25.0% smokers). DR was present only in patients with T1DM+CP, and DPN was also present significantly more frequently in this group of patients ($P < 0.01$).

Table 1. Demographic data for the studied T1DM+CP and T2DM+CP patients as well as HC+CP and non-periodontitis HC.

Characteristics (mean ± SD)	Non-Periodontitis HC *N* = 32	HC+CP *N* = 41	T1DM+CP *N* = 36	T2DM+CP *N* = 44
Age (years)	45.5 ± 9.6	59.5 ± 9.3 *	49.7 ± 10.4	66.8 ± 8.5 *
Duration of DM (years)	0.0 ± 0.0	0.0 ± 0.0	25.1 ± 11.8	10.2 ± 7.7
DM control (well/satisfactorily/ unsatisfactorily controlled) %	–	–	0.0/8.3/91.7	7.7/28.2/64.1
Sex (males) %	31.3	26.8	44.4	40.9
Smoking (yes) %	5.3	19.4	25.0 *	7.1 ‡,§
DN (yes) %	0.0	0.0	27.6	16.7
DR (yes) %	0.0	0.0	63.3 ‖	0.0
DPN (yes) %	0.0	0.0	50.0 ‖	9.3
BMI	23.8 ± 4.2	26.8 ± 3.9	25.1 ± 3.1	29.9 ± 7.7 *,§
GI	0.3 ± 0.2	0.9 ± 0.3 †	1 ± 0.3 †	1.1 ± 0.3 †
PD (mm)	0.6 ± 0.2	3.3 ± 0.9 *	3.6 ± 0.9 †	3.8 ± 1.0 †
AL (mm)	0.8 ± 0.2	4.1 ± 1.1 †	4.4 ± 1.5 †	4.7 ± 1.2 †
N of sites with PD ≥ 5 mm	0	18 ± 17 *	20 ± 19 *	20 ± 16 *
N of teeth with PD ≥ 5 mm	0	10 ± 7 *	11 ± 7 *	11 ± 6 *
N of sites with AL ≥ 5 mm	0	32 ± 21 *	38 ± 28 *	38 ± 19 *
N of teeth with AL ≥ 5 mm	0	15 ± 7 *	15 ± 7 *	15 ± 6 *
HbA1c (mmol/mol)	-	-	69.6 ± 12.0	57.7 ± 14.5 §
Blood glucose (mmol/L)	5.4 ± 0.5	5.7 ± 0.3	7.5 ± 2.3	7.7 ± 2.4
Total cholesterol (mmol/L)	-	-	4.7 ± 0.7	4.7 ± 1.0
Triglycerides (mmol/L)	-	-	1.0 ± 0.6	2.0 ± 1.2
LDL (mmol/L)	-	-	2.7 ± 0.6	2.7 ± 0.9
HDL (mmol/L)	-	-	1.6 ± 0.4	1.3 ± 0.3

– = unknown. Note: In T1DM+CP patients, the diabetic control was known only in 31 of them. * $P < 0.05$ in comparison to non-periodontitis HC. † $P < 0.01$ in comparison to non-periodontitis HC. ‡ $P < 0.05$ in comparison to HC+CP individuals. § $P < 0.05$ in comparison to T1DM+CP patients. ‖ $P < 0.01$ in comparison to T2DM+CP patients. DM: diabetes mellitus; DN: diabetic nephropathy; DR: diabetic retinopathy; DPN: diabetic neuropathy; BMI: body mass index; GI: gingival index; PD: pocket depth; AL: attachment loss; LDL: low density lipoprotein; HDL: high density lipoprotein.

There were statistically significant differences between non-periodontitis HC and all of the subgroups of cases in gingival index (GI) and numbers (*N*) of sites and teeth with a pocket depth (PD) ≥5 mm and an attachment loss (AL) ≥5 mm ($P < 0.01$). Nevertheless, similar numbers were found in mutual comparisons of HC+CP, T1DM+CP, and T2DM+CP patient groups ($P > 0.05$). Although the groups of diabetic patients had similar lipid profiles and blood glucose levels, T1DM+CP patients had poorly controlled glycated hemoglobin (HbA1c) levels when compared to T2DM+CP patients (69.6 ± 12.0 vs. 57.7 ± 14.5, $P < 0.05$). The group of patients with T1DM+CP included only three patients with satisfactory DM control. DM control in other patients was unsatisfactory as measured by HbA1c values. The stratification in the group of patients with T2DM+CP was as follows: 7.7% of patients with well-controlled diabetes, 28.2% with satisfactorily controlled diabetes, and 64.1% with unsatisfactorily controlled diabetes.

2.2. IL-8 Plasma Levels and Clinical Parameters

Analysis of circulating IL-8 showed that plasma levels of this chemokine differed significantly between the T1DM+CP/T2DM+CP patients (median [interquartile range, IQR]: 15.09 pg/mL [9.73–20.32]/14.25 pg/mL [11.72–23.36], respectively) and non-periodontitis HC (10.53 pg/mL [8.48–12.58], $P < 0.01$ in both comparisons). Diabetic patients of both types with CP also had significantly higher levels of IL-8 than did HC+CP individuals (11.02 pg/mL [6.47–15.17], $P \leq 0.05$). Interestingly, the groups of HC+CP and non-periodontitis HC and also the groups of patients with T1DM+CP and T2DM+CP always exhibited similar IL-8 plasma levels (see Figure 1).

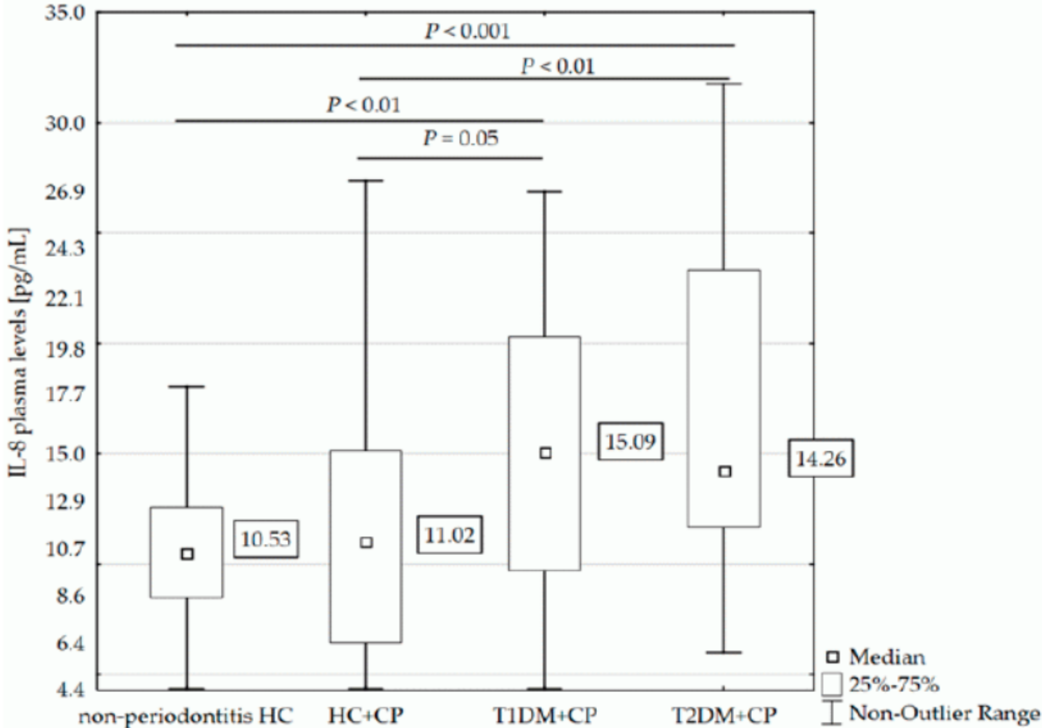


Figure 1. Comparison of IL-8 plasma levels in T1DM+CP ($N = 36$) and T2DM+CP patients ($N = 44$) and HC+CP ($N = 41$) and non-periodontitis HC ($N = 32$). Samples with IL-8 levels under the limit of detection (<4.40 pg/mL) were assigned a value of 4.39 pg/mL. The Mann-Whitney U-test (two-tail) was used for the calculation of significant differences.

The IL-8 plasma levels in diabetic patients according to their glycemic control (as assessed by HbA1c levels) were comparable ($P > 0.05$, see Table 2).

Table 2. Comparison of IL-8 plasma levels in diabetic patients according to their glycemic control.

IL-8 Levels, Median [IQR] in pg/mL		
Well Controlled $N = 4$	Satisfactorily Controlled $N = 14$	Unsatisfactorily Controlled $N = 57$
12.68 [10.52–40.56]	14.45 [12.15–23.13]	14.04 [10.05–19.67]

Note: In T1DM+CP patients, the diabetic control was known only in 31 of them. IQR: interquartile range.

The comparison of IL-8 levels in T2DM patients of normal weight, those who were overweight, and obese showed no significant differences ($P > 0.05$, see Table 3).

Table 3. Comparison of IL-8 plasma levels in T2DM patients according to their BMI.

IL-8 Levels, Median [IQR] in pg/mL		
Normal Weight BMI ≤ 25.0 kg/m^2 $N = 9$	Overweight 25 kg/m^2 < BMI ≤ 30 kg/m^2 $N = 14$	Obese BMI > 30 kg/m^2 $N = 21$
12.68 [9.16–16.54]	13.75 [12.00–23.13]	14.04 [12.50–16.24]

IQR: interquartile range.

In addition, concentrations of circulating IL-8 levels were not significantly associated with the level of glycemic control (blood glucose and HbA1c), smoking status, or clinical parameters such as GI, PD and AL ($P > 0.05$). However, increased IL-8 levels were detected in patients without DN (14.84 pg/mL [11.60–21.79]) in comparison to patients with DN (12.33 pg/mL [7.84–15.51]), $P = 0.03$).

2.3. IL-8 Plasma Levels and Gene Variability

The frequencies of both studied single nucleotide polymorphisms (SNPs), *IL-8* (−251A/T, rs4073) and *CXCR2* (+1208C/T, rs1126579) genotypes, in all subgroups were consistent with those expected from the Hardy-Weinberg equilibrium (HWE) ($P > 0.05$).

Allelic and genotype distributions were similar in all of the subgroups and the T2DM+CP group between well/satisfactorily controlled individuals vs. unsatisfactorily controlled patients (only data for genotype frequencies are shown). *IL-8* and *CXCR2* gene variability was also evaluated across the whole cohort in relation to IL-8 plasma levels. No association between the specific gene variants and circulating IL-8 levels was found (see Table 4).

Table 4. IL-8 plasma levels and polymorphisms in *IL-8* and *CXCR2* genes *.

SNPs Genotypes		IL-8 Levels, Median [IQR] in pg/mL											
		Non-Periodontitis HC N = 32 (%)		HC+CP N = 41 (%)		T1DM+CP N = 36 (%)		T2DM+CP N = 43 (%)		T2DM+CP Subgroups			
										Well/Satisfactorily Controlled N = 16 (%)		Unsatisfactorily Controlled N = 27 (%)	
IL-8 (−251A/T, rs4073)	TT	10 (31.3)	9.20 [8.61–11.86]	10 (24.4)	10.36 [6.47–13.60]	11 (30.6)	13.16 [10.43–19.67]	16 (37.2)	13.75 [10.73–25.39]	5 (31.3)	12.87 [12.29–24.63]	11 (40.7)	14.33 [9.16–26.14]
	AT	15 (46.9)	11.00 [7.52–12.87]	21 (51.2)	7.68 [5.66–15.17]	16 (44.4)	17.38 [7.01–20.92]	17 (39.5)	14.18 [11.43–16.54]	6 (37.4)	15.10 [9.86–20.27]	11 (40.7)	13.49 [11.43–16.54]
	AA	7 (21.8)	10.86 [8.61–12.66]	10 (24.4)	12.19 [8.88–16.24]	9 (25.0)	13.33 [10.86–22.70]	10 (23.3)	15.80 [12.33–23.13]	5 (31.3)	16.69 [15.36–23.13]	5 (18.6)	14.04 [12.00–16.24]
CXCR2 (+1208C/T, rs1126579)	CC	9 (28.1)	11.28 [8.61–12.87]	14 (34.1)	12.30 [7.92–15.21]	9 (25.0)	17.89 [10.43–20.87]	17 (39.5)	15.09 [12.29–23.91]	7 (43.8)	15.36 [12.29–24.63]	10 (37.0)	14.88 [9.16–23. 91]
	CT	17 (53.1)	9.09 [7.52–11.43]	18 (43.9)	11.57 [6.47–15.17]	20 (55.6)	14.00 [8.63–23.52]	21 (48.9)	13.16 [11.43–16.24]	7 (43.8)	14.18 [9.86–30.99]	14 (51.9)	13.16 [11.43–16.24]
	TT	6 (18.8)	11.43 [10.86–12.50]	9 (22.0)	6.99 [5.66–11.02]	7 (19.4)	12.00 [4.39–18.57]	5 (11.6)	16.69 [13.49–20.27]	2 (12.4)	18.48 [16.69–20.27]	3 (11.1)	13.49 [12.00–31.74]

* Genotypes are known only in 152 participants as one DNA sample was of poor quality.

2.4. IL-8 Plasma Levels and Periodontal Bacteria

The microbial profiles were determined in a subgroup of 140 subjects. The participants were divided into two groups (negative and positive) for specific periodontal bacteria. Higher IL-8 plasma levels were found in the healthy controls and the well/satisfactorily controlled T2DM+CP patients positive for *T. forsythia* or *P. intermedia* than in individuals from this subgroup negative for these specific bacteria ($P = 0.03$ in both, $P_{corr} > 0.05$). On the other hand, the presence of *P. gingivalis, T. forsythia*, or *T. denticola* was associated with lower IL-8 plasma levels in T1DM+CP patients ($P < 0.01$, $P_{corr} > 0.05$).

F. nucleatum was detected in almost all of the individuals (98.6%). IL-8 levels were found to be higher in the absence of *F. nucleatum* than in the presence of this bacterium in HC+CP individuals (mean 46.40 pg/mL vs. 10.80 pg/mL), and in unsatisfactorily controlled T2DM+CP patients (mean 30.86 pg/mL vs. 13.77 pg/mL). However, the significance of these results is questionable due to the low number of patients who tested negative for *F. nucleatum* (only two individuals in total; see Table 5).

Table 5. IL-8 plasma levels and periodontal bacteria in a subgroup of 140 individuals.

Bacteria (%)	IL-8 Levels, Median [IQR] in pg/mL											
	Non-Periodontitis HC $N = 19$		HC+CP $N = 41$		T1DM+CP $N = 36$		T2DM+CP $N = 44$		T2DM+CP Subgroups			
									Well/Satisfactorily Controlled $N = 17$		Unsatisfactorily Controlled $N = 27$	
F. n. neg	0.0	-	2.4	46.40 [46.40–46.40]	0.0	-	2.3	30.86 [30.86–30.86]	0.0	-	3.7	30.86 [30.86–30.86]
F. n. pos	100.0	10.21 [8.61–12.66]	97.6	10.80 [6.43–14.83]	100.0	15.09 [9.73–20.32]	97.7	14.18 [11.43–23.13]	100.0	15.36 [12.29–23.13]	96.3	13.77 [11.43–16.54]
A. a. neg	68.4	10.21 [8.61–12.50]	48.8	7.80 [5.99–13.32]	55.6	18.41 [10.38–24.41]	38.6	13.49 [12.33–16.54]	29.4	28.16 [12.87–30.99]	44.4	13.33 [10.58–14.50]
A. a. pos	31.6	10.14 [9.09–13.49]	51.2	13.45 [8.75–15.17]	44.4	13.25 [8.63–17.72]	61.4	15.09 [11.18–23.58]	70.6	14.77 [10.52–18.48]	55.6	15.09 [12.00–26.14]
P. g. neg	68.4	9.73 [7.24–12.66]	14.6	13.18 [7.99–27.35]	25.0	24.33 [16.86–24.78]	22.7	14.26 [12.33–26.14]	17.6	12.33 [9.09–15.36]	25.9	16.24 [12.50–30.86]
P. g. pos	31.6	10.54 [9.41–12.50]	85.4	10.57 [6.02–14.48]	75.0	12.00 [7.24–18.92] *	77.3	14.26 [11.43–23.13]	82.4	16.36 [12.29–24.63]	74.1	13.77 [10.58–15.82]
T. f. neg	36.8	8.61 [6.72–9.73]	2.4	46.40 [46.40–46.40]	8.3	24.33 [19.67–40.67]	0.0	-	0.0	-	0.0	-
T. f. pos	63.2	11.67 [9.81–13.66] *	97.6	10.80 [6.43–14.83]	91.7	13.33 [9.41–19.43] *	100.0	14.26 [11.72–23.36]	100.0	15.36 [12.29–23.13]	100.0	14.04 [11.43–23.58]
T. d. neg	47.4	8.77 [7.24–12.33]	14.6	14.83 [13.45–19.37]	25.0	20.87 [19.43–24.78]	22.7	13.33 [12.50–15.36]	17.6	12.87 [12.33–15.36]	25.9	13.49 [12.50–16.54]
T. d. pos	52.6	10.93 [9.41–13.83]	85.4	10.15 [6.39–14.04]	75.0	12.00 [7.24–18.57] *	77.3	14.50 [11.18–23.91]	82.4	16.36 [11.18–24.63]	74.1	14.19 [10.58–23.75]
P. m. neg	31.6	9.89 [7.24–12.66]	2.4	46.40 [46.40–46.40]	0.0	-	0.0	-	0.0	-	0.0	-
P. m. pos	68.4	10.21 [9.09–12.50]	97.6	10.80 [6.43–14.83]	100.0	15.09 [9.73–20.32]	100.0	14.26 [11.72–23.36]	100.0	15.36 [12.29–23.13]	100.0	14.04 [11.43–23.58]
P. i. neg	47.4	8.77 [6.72–12.66]	41.5	7.99 [6.02–14.48]	41.7	17.89 [12.15–24.33]	45.5	14.08 [10.14–16.39]	35.3	11.76 [9.09–15.36]	51.9	15.05 [11.43–23.58]
P. i. pos	52.6	10.54 [9.41–12.50]	58.5	11.57 [7.60–15.19]	58.3	12.00 [7.84–18.92]	54.5	14.26 [12.40–24.27]	64.7	20.27 [12.87–28.16] *	48.1	13.49 [12.00–14.67]

Neg: negative; pos: positive. * $P < 0.05$ in comparison of IL-8 plasma levels between patients negative and positive for the specific bacteria ($P_{corr} > 0.05$).

3. Discussion

CP and DM are common, multifactorial diseases worldwide [31]. Current evidence suggests that the relationship is bidirectional: DM increases the risk and severity of periodontitis, and periodontal disease can adversely affect the outcome of diabetes [32]. A potential link between DM and CP involves a broad axis of inflammation, a specific immune cell phenotype, serum lipid levels, and tissue homeostasis [33]. Changes in immune cell function in diabetic patients are reflected in the upregulation of proinflammatory cytokines and chemokines, such as the neutrophils chemoattractant IL-8 [34].

In this study, we evaluated the circulating levels of IL-8 as well as genetic and microbial profiles in systemically healthy individuals with or without CP and T1DM/T2DM patients with CP. The participants were selected from a large pool of patients and controls; in all groups, representation of both genders was balanced. The average ages of the non-periodontitis HC and T1DM+CP patients and also HC+CP and T2DM+CP patients were similar. The difference in the average age between patients with T1DM and T2DM was caused by divergent pathogenesis (age of onset) of the two types of the disease. Nevertheless, patients with T1DM were affected on average by a 15-year longer duration of this disease compared to those with type 2. Simultaneously, patients with T1DM+CP had more poorly controlled HbA1c levels than T2DM+CP patients. Periodontitis progression has recently been associated with elevated HbA1c levels in T2DM patients, and the treatment of periodontal infection may thus improve glycemic control of diabetic patients [35]. In this study, however, periodontal status, evaluated according to PD and AL, was similar between the CP patients and DM patients of both types with CP. Therefore, our results are in contradiction to other published articles which conclude that diabetic patients with difficulties in controlling serum glucose levels are more likely to suffer from periodontitis than well-controlled diabetic patients or individuals without diabetes [36]. This may be due to the relatively small sample size included in this study.

Based on the finding that high glucose-induced oxidative stress increases IL-8 production in human gingival epithelial cells, Kashiwagi et al. hypothesized a potential involvement of epithelial cells in periodontal disease during diabetes, caused by evoking an excessive host inflammatory response [22]. We found that IL-8 plasma levels were significantly higher in both diabetic groups than in HC+CP/non-periodontitis HC individuals. We assume that the differences in mean age between the groups did not affect the results. Even though IL-8 plasma levels were previously found higher in healthy older people than in healthy younger people, there was no statistical significance of this result [37]. Recent studies have shown that IL-8 is secreted by adipocytes, that circulating IL-8 levels in obese subjects without diabetes are significantly higher than in subjects with normal body weight, and that circulating levels of IL-8 are thus positively correlated with BMI [38]. In this study, no significant differences were found in IL-8 levels among T2DM normal weight, overweight, or obese subjects. However, this can be due to the low number of T2DM patients with normal body weight. While Engebretson et al. and Mohamed et al. reported significantly higher concentrations of IL-8 in GCF in patients with T2DM+CP as compared to HC+ CP [23,25], IL-8 GCF levels did not correlate with the diabetic status in the recent study by Longo et al. [24]. In contrast to our results, IL-8 circulating levels were found to be significantly lower in patients with T1DM than in first-degree control relatives [26]. Purohit et al. hypothesized that higher levels of this cytokine might be partly responsible for the protection against the development of T1DM [26]. On the other hand, Lappin et al. also found elevated plasma levels of IL-8 in T1DM+CP patients [21], and thus IL-8 may contribute to the cross-susceptibility between CP and DM. In addition, plasma levels of IL-8 were found to be similar in non-periodontitis HC and the HC+CP individuals in the Czech population. Nevertheless, in previous studies, higher plasma/serum levels have been associated with CP in different populations [21,39]. Frederiksson suggested that patients with CP had a subpopulation of peripheral neutrophils with a higher responsiveness to IL-8 priming than controls with healthy gingiva [40].

No association was found between circulating IL-8 level and glycemic control (blood glucose and HbA1c), smoking status, and clinical parameters such as PD and AL in the Czech population. In contrast, Lappin et al. reported that plasma levels of IL-8 correlated with levels of blood glucose

and HbA1c, PD, and AL [21]. It should be noted that mean numbers of sites/teeth with PD/AL ≥ 5 mm in Czech patients were more than double of those in patients with CP and T1DM+CP in the study conducted by Lappin et al. [21]. The severity of periodontal tissue damage and inflammation may thus contribute to the differences in results. Surprisingly, higher IL-8 levels in patients without DN than in patients with DN were found in the studied cohort. This is not in accordance with the study by Perlman et al., who found an elevated transcript level of IL-8 at all DN disease stages as compared to controls [41]. Our results must be treated with caution because the number of patients with DN was very low (only 17 patients).

A further aim of this study was to correlate IL-8 circulating levels with variants in the *IL-8* and *CXCR2* genes. *IL-8* polymorphisms (−251A/T, rs4073) had previously been associated with changes in transcriptional activity [42]; namely, the A allele was linked with an increased IL-8 production in response to whole blood stimulation with lipopolysaccharides (LPS) [27]. In line with the results presented by Li et al. [39], no correlation of IL-8 plasma levels with the gene variants in *IL-8* was found in this study. We assume that no individual SNPs in the *IL-8* gene, but rather a combination of multiple variants, may affect the protein expression. Benakanakere et al. provide evidence that carriage of the *IL-8* ATC/TTC haplogenotype may increase the influx of neutrophils into inflammatory lesions and influence disease susceptibility [43]. Although synergistic interaction was observed between the T allele of the *CXCR2* (+1208C/T, rs1126579) SNP and high IL-8 serum levels [29], there were no differences among single variants and plasma levels of IL-8 in the present study. The current results of the genetic analysis were analogous with those from our previous research into *IL-8* gene variability in CP and aggressive periodontitis patients in a larger population [30]. Recent work by Nibali et al. claimed that host genetic variants could affect the colonization by specific microbes [44]. However, this could not be confirmed in this study due to the small size of the studied population.

Finally, the relationship between the microbial profile and chemokine levels was evaluated, but the presence of individual periodontopathic bacteria was not significantly associated with IL-8 production. Mesia et al. demonstrated that T2DM+CP individuals had higher unstimulated and stimulated levels of several cytokines, including IL-8, than systemically healthy individuals with periodontal disease [45]. In addition, *P. gingivalis* LPS-induced levels of IL-8 and others strongly correlated with disease severity (as measured by PD) in the T2DM group, but not in the group of controls with periodontitis [45]. While Lappin et al. found that *P. gingivalis* LPS and AGE together influenced the expression of IL-8 more than LPS alone in vitro [21], there were no differences in the presence of this bacterium between systemically healthy individuals and DM patients with similar periodontal status in the Czech population. Interestingly, *F. nucleatum* was detected in 98.6% of participants and higher IL-8 levels were recorded in patients without this bacterium in the oral cavity. This is inconsistent with previous findings, which showed that various *F. nucleatum* strains produce higher IL-8 levels in comparison with other oral bacteria [46,47]. The mechanisms behind these observations are still not well understood. Furthermore, our study only found two patients who were negative for *F. nucleatum*, one was from the group HC+CP and the other suffered from T2DM+CP.

The main limitation of our study is that subgroups of T1DM/T2DM without periodontal disease could not be included because only diabetic patients with a different severity of periodontitis were in the selected cohort. On the other hand, we can claim high homogeneity of the studied population because the participants were collected from a pool of patients of Czech Caucasians of European origin from South Moravia. The size of our cohort (N = 109) is comparable with the number of participants (N = 104) in the recent study by Lappin et al. [21]; moreover, we included an additional 44 T2DM+CP patients. Lappin et al. associated the elevated plasma levels of IL-8 with CP and/or T1DM and found that *P. gingivalis* LPS and AGE together caused a significantly greater expression of IL-8 from THP-1 monocytes and OKF6/TERT-2 cells than LPS alone. While no in vitro stimulation of cells by bacterial LPS or AGE was carried out in connection with IL-8 in our previous study [48], the advantage of the present study is that the Czech participants underwent detailed clinical, genetic, and microbiological examinations.

In conclusion, increased circulating levels of IL-8 were associated with DM of both types in the presence of periodontal disease, which suggests their important role in the pathogenesis of T1DM, T2DM, and CP. Nevertheless, the influence of single genetic variants of *IL-8/CXCR2* or the influence of the presence of individual periodontal bacteria on the concentrations of circulating IL-8 were not confirmed. Our findings may have some diagnostic implications and their molecular basis needs to be further addressed. Nevertheless, the design of the association study can be sensitive to type II statistical error and our results require confirmation by other studies on different populations.

4. Materials and Methods

The study was performed with the approval of the Committees for Ethics (15/2009) of the Medical Faculty, Masaryk University Brno and St. Anne's Faculty Hospital. Written informed consent was obtained from all participants in line with the Declaration of Helsinki before inclusion into the study.

4.1. Study Population and Clinical Examinations

One hundred and fifty-three unrelated Caucasian adult participants from the South Moravian Region of the Czech Republic were included in this study. The periodontal status was evaluated in all individuals as follows: 73 systemically healthy individuals and 80 diabetic patients at the Clinic of Stomatology, Institution Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Brno from 2010 to 2017. The diagnosis of periodontitis/non-periodontitis was based on detailed clinical examination, medical and dental history, tooth mobility and radiographic assessment. All patients with CP fulfilled the diagnostic criteria defined according to AL levels by the International Workshop for a Classification of Periodontal Diseases and Conditions for Chronic Periodontitis [49]: $\geq$30% of the teeth were affected (generalized CP) and PD was $\geq$4 mm. PD and AL were collected with a UNC-15 periodontal probe from four sites on every tooth present by an experienced periodontist.

For comparability with the study by Lappin et al. [21], the numbers of sites and teeth with PD $\geq$ 5 mm and AL $\geq$ 5 mm were recorded in both cases and controls. Loss of the alveolar bone was determined radiographically. Examination of lipid level profiles was as previously described [50]. The degree of gingival inflammation was assessed using the GI according to Löe and Silness [51], and the presence of inflammation of the gingiva was evaluated on four surfaces of all of those teeth present (distal, vestibular, mesial, oral). This index uses a 0–3 scale according to the following criteria: The complete absence of visual signs of inflammation was scored as 0; slight change in colour, slight oedema and no bleeding on probing as 1; and visual inflammation, redness, oedema, glazing, and bleeding on pressure as 2. Finally, severe inflammation, marked redness, oedema, ulceration, and the tendency to spontaneous bleeding was scored as 3. Using all of the individual scores, mean GI scores $\pm$ SD were calculated.

The diagnosis of T1DM (number of patients, N = 36) or T2DM (N = 44) was based on the presence of clinical symptoms (such as polyuria, polydipsia, and weight loss) and biochemical parameters (glycemia, glycated hemoglobin, ketoacidosis, and autoantibody status) in the outpatient unit of the Diabetology Clinics in Brno by experienced diabetologists. In accordance with American Diabetes Association guidelines [52], in patients with typical symptoms, the diagnosis was established upon finding glucose >11.0 mmol/L. In the absence of clinical manifestations, the diagnosis was made based on the finding of fasting blood glucose in venous plasma $\geq$7.0 mmol/L after 8 h of fasting, and the finding of blood glucose in venous plasma >11.0 mmol/L, or HbA1c $\geq$ 48 mmol/mol 2 h after the consumption of 75 g glucose (oral glucose tolerance test). Diabetes control was assessed as good (<45 mmol/mol), satisfactory (45–60 mmol/mol), or unsatisfactory (>60 mmol/mol) [53]. In addition, the presence of diabetic complications (such as DR, DPN, and DN), the duration of diabetes, and other clinical and biochemical parameters (BMI, smoking, lipid profile, etc.) were recorded.

The inclusion criteria for this study were the willingness to participate, compliance with the diagnostic criteria for CP and/or DM, and, for the control group, systemic and periodontal health. None of the participants were receiving treatment for periodontitis at the time of diagnosis but all

were offered treatment whether they agreed, declined to participate, or were excluded from the study. All patients were firstly examined by a periodontist and they did not receive scaling and/or root planing minimally six months before measuring periodontal indices. The exclusion criteria for this study were a history of systemic diseases such as coronary artery diseases, malignancies, immunodeficiency disorders, current pregnancy or lactation, immunosuppression attributable to medication or concurrent illness, the use of antibiotics or anti-inflammatory drugs within six weeks of recruitment, <20 teeth (only in healthy controls), and the inability to consent.

4.2. Sample Collection and Plasma Levels Analysis

Levels of IL-8 in plasma were measured in all 153 individuals at the Department of Biochemistry, Faculty of Medicine, Masaryk University, Brno. The plasma samples were prepared from venous blood collected into a tube with EDTA (S-Monovette® 9 mL K3E, Sarstedt, Germany), separated by centrifugation (465× *g*, 4 °C, 10 min), and stored at −70 °C within 30 min of collection.

IL-8 plasma levels were determined using enzyme-linked immunosorbent assay (ELISA) kits [IL-8 Human Magnetic Kit for Luminex™ Platform (Catalog No. LHC0081M, Novex™, Life Technologies, Grand Island, NY, USA) with Human/Monkey Extracellular Protein Buffer Magnetic Reagent Kit (Catalog No. LHB0001M, Novex™, Life Technologies, Grand Island, NY, USA)] and software (Luminex 200™ analyzer with xPONENT 3.1 Software, Luminex Corporation, USA; Milliplex™ Analyst v 3.4 Software, VigeneTech, Carlisle, MA USA) according to the manufacturer's instructions. Those samples with IL-8 values under the limit of detection (<4.40 pg/mL) were arbitrarily assigned a value of 4.39 pg/mL for the statistical analysis.

4.3. Genetic Analysis

Genomic DNA was isolated from peripheral blood according to the standard protocol (phenol-chloroform method) by Sambrook et al. [54] and archived in the DNA bank at the Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno.

Two SNPs [*IL-8* (−251A/T, rs4073) and *CXCR2* (+1208C/T, rs1126579)] were genotyped using the fluorescent probes for allelic discrimination (TaqMan® assays, Life Technologies, Grand Island, NY, USA; C_11748116_10 and C_8841198_10, respectively). A sequence detection system ABI PRISM 7000, Applied Biosystems, Waltham, MA, USA was used. Polymerase chain reaction conditions were 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Real-time and endpoint fluorescence data were analyzed by SDS version 1.2.3, Applied Biosystems, USA software. Ten percent of the samples were determined in duplicates with 100% accordance. Genotyping was performed by investigators unaware of the phenotype.

4.4. Periodontal Bacteria Analysis

Samples of subgingival microflora were collected from the deepest sulcus/pocket from each quadrant in oral cavity. After careful removal of supragingival plaque and drying, a sterile endodontic pin (ISO size 40) was introduced to the bottom of the periodontal sulcus for 10 s. After removal, the pins were inserted into a sterile transport tube and sent for evaluation.

Seven oral bacteria were investigated by the DNA microarray detection kit (Protean Ltd., Ceske Budejovice, Czech Republic), as published previously [30]. Briefly, individual bacteria were determined semi-quantitatively as follows: (−) undetected, which corresponds to bacteria count less than 10^3; (+) slightly positive, corresponding to bacteria count of 10^3–10^4; (++) positive, corresponding to bacteria count of 10^4–10^5; and (+++) strongly positive, with bacteria count higher than 10^5. Subgingival bacterial colonization (*Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans, P. gingivalis, T. forsythia, Treponema denticola, Prevotella intermedia, Parvimonas micra*) in subgingival sulci/pockets was analyzed in the subgroups of non-periodontitis HC (N = 19), HC+CP (N = 41), T1DM+CP (N = 36) and T2DM+CP patients (N = 44) before subgingival scaling. The diagnosis of the specific bacterial infection was assessed as positive when the number of bacterial cells exceeded 10^3.

4.5. Statistical Analysis

In accordance with the "case–control" design of this study, "controls" (systemically healthy people without CP, so called non-periodontitis HC) were compared with "cases" (all patients with CP regardless of their diabetes status: HC+CP, T1DM+CP or T2DM+CP patients). Secondly, we used a "case–case" design to compare systemically healthy individuals and T1DM or T2DM patients with a similar periodontal status, as well as to make comparisons between patients with both types of diabetes.

Standard descriptive statistics were calculated: absolute and relative frequencies for categorical variables, mean with SD or median with quartiles for quantitative variables. One–way analysis of variance (ANOVA) and Kruskal–Wallis test (ANOVA) were carried out to compare continuous variables. The allele frequencies were calculated from the observed numbers of genotypes. The differences in the allele frequencies were tested using the Fisher's exact test; HWE and genotype frequencies were calculated with the chi-square test. The association was described by odds ratios with 95% confidence intervals. Only the values of $P < 0.05$ were considered as statistically significant. Where appropriate, Bonferroni correction was used to adjust the level according to the number of independent comparisons to the overall value of 0.05. The adjusted P values are denoted as P_{corr}. Statistical power calculation was based on the results from the previous study on plasma IL-8 levels by Lappin et al. [21]. To obtain an excess of 80% statistical power in an ANOVA with a detectable difference of 1.2 and mean of 1, a minimum of 17 samples was required in each of the four patient groups [21]. Similar to the previous study, we also increased the number to a minimum of 19 per group because the data did not conform to the normal distribution. Statistical analysis was performed using the statistical package Statistica v. 12, StatSoft Inc., Tulsa, OK, USA.

Author Contributions: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Data curation, Visualization, Writing—original draft preparation, P.B.L.; Writing—review and editing, D.K.; Methodology, Writing—review and editing, M.T., H.P. and V.R.; Software, Validation, L.D.; Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing—review and editing, Supervision, L.I.H.

Funding: This study was supported by the grants GACR GB14-37368G, the project MUNI/A/1008/2017, and funds from the Faculty of Medicine of Masaryk University awarded to junior researcher Petra Borilova Linhartova.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Abbreviations

A. a.	*Aggregatibacter actinomycetemcomitans*
AL	Attachment loss
ANOVA	One-way analysis of variance
BMI	Body mass index
CXCR2	C-X-C motif chemokine receptor 2 (receptor for IL-8)
CP	Chronic periodontitis
DM	Diabetes mellitus
DN	Diabetic nephropathy
DPN	Diabetic neuropathy
DR	Diabetic retinopathy
ELISA	Enzyme-linked immunosorbent assay
F. n.	*Fusobacterium nucleatum*
GCF	Gingival crevicular fluid
GI	Gingival index
HbA1c	Glycated hemoglobin
HC	Systemically healthy controls without periodontitis

HC+CP	Systemically healthy individuals with chronic periodontitis
HDL	High density lipoprotein
HWE	Hardy-Weinberg equilibrium
IL-8	Interleukin-8
IQR	Interquartile range
LDL	Low density lipoprotein
LPS	lipopolysaccharide
N	Number
Neg	Negative
PD	Pocket depth
P. g.	*Porphyromonas gingivalis*
P. i.	*Prevotella intermedia*
P. m.	*Parvimonas micra*
Pos	Positive
qPCR	Quantitative polymerase chain reaction
SD	Standard deviation
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
T. d.	*Treponema denticola*
T. f.	*Tanarella forsythia*
P. i.	*Prevotella intermedia*

References

1. Harris, S.B.; Tompkins, J.W.; TeHiwi, B. Call to action: A new path for improving diabetes care for Indigenous peoples, a global review. *Diabetes Res. Clin. Pract.* **2017**, *123*, 120–133. [CrossRef] [PubMed]
2. Deshpande, A.D.; Harris-Hayes, M.; Schootman, M. Epidemiology of diabetes and diabetes-related complications. *Phys. Ther.* **2008**, *88*, 1254–1264. [CrossRef] [PubMed]
3. Chawla, D.; Bansal, S.; Banerjee, B.D.; Madhu, S.V.; Kalra, O.P.; Tripathi, A.K. Role of advanced glycation end product (AGE)-induced receptor (RAGE) expression in diabetic vascular complications. *Microvasc. Res.* **2014**, *95*, 1–6. [CrossRef] [PubMed]
4. Papatheodorou, K.; Banach, M.; Edmonds, M.; Papanas, N.; Papazoglou, D. Complications of diabetes. *J. Diabetes Res.* **2015**, *2015*, 189525. [CrossRef] [PubMed]
5. Löe, H. Periodontal disease. The sixth complication of diabetes mellitus. *Diabetes Care* **1993**, *16*, 329–334. [CrossRef] [PubMed]
6. Acharya, A.B.; Thakur, S.; Muddapur, M.V.; Kulkarni, R.D. Systemic cytokines in type 2 diabetes mellitus and chronic periodontitis. *Curr. Diabetes Rev.* **2018**, *14*, 182–188. [CrossRef] [PubMed]
7. Genco, R.J. Consensus report. Periodontal diseases: Pathogenesis and microbial factors. *Ann. Periodontol.* **1996**, *1*, 926–932. [CrossRef]
8. Armitage, G.C. Comparison of the microbiological features of chronic and aggressive periodontitis. *Periodontol. 2000* **2010**, *53*, 70–88. [CrossRef] [PubMed]
9. Nibali, L.; Henderson, B.; Sadiq, S.T.; Donos, N. Genetic dysbiosis: The role of microbial insults in chronic inflammatory diseases. *J. Oral Microbiol.* **2014**, *6*, 22962. [CrossRef] [PubMed]
10. Festa, A.; D'Agostino, R., Jr.; Howard, G.; Mykkänen, L.; Tracy, R.P.; Haffner, S.M. Chronic subclinical inflammation as part of the insulin resistance syndrome: The Insulin Resistance Atherosclerosis Study (IRAS). *Circulation* **2000**, *102*, 42–47. [CrossRef] [PubMed]
11. Moutsopoulos, N.M.; Madianos, P.N. Low-grade inflammation in chronic infectious diseases: Paradigm of periodontal infections. *Ann. N. Y. Acad. Sci.* **2006**, *1088*, 251–264. [CrossRef] [PubMed]
12. Manouchehr-Pour, M.; Spagnuolo, P.J.; Rodman, H.M.; Bissada, N.F. Comparison of neutrophil chemotactic response in diabetic patients with mild and severe periodontal disease. *J. Periodontol.* **1981**, *52*, 410–415. [CrossRef] [PubMed]
13. Wuyts, A.; Proost, P.; Lenaerts, J.P.; Ben-Baruch, A.; Van Damme, J.; Wang, J.M. Differential usage of the CXC chemokine receptors 1 and 2 by interleukin-8, granulocyte chemotactic protein-2 and epithelial-cell-derived neutrophil attractant-78. *Eur. J. Biochem.* **1998**, *255*, 67–73. [CrossRef] [PubMed]

14. Harada, A.; Sekido, N.; Akahoshi, T.; Wada, T.; Mukaida, N.; Matsushima, K. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J. Leukoc. Biol.* **1994**, *56*, 559–564. [CrossRef] [PubMed]
15. Ahmadi, Z.; Arababadi, M.K.; Hassanshahi, G. CXCL10 activities, biological structure, and source along with its significant role played in pathophysiology of type I diabetes mellitus. *Inflammation* **2013**, *36*, 364–371. [CrossRef] [PubMed]
16. Ismail, N.A.; Abd El Baky, A.N.; Ragab, S.; Hamed, M.; Hashish, M.A.; Shehata, A. Monocyte chemoattractant protein 1 and macrophage migration inhibitory factor in children with type 1 diabetes. *J. Pediatr. Endocrinol. Metab.* **2016**, *29*, 641–645. [CrossRef] [PubMed]
17. Harsunen, M.H.; Puff, R.; D'Orlando, O.; Giannopoulou, E.; Lachmann, L.; Beyerlein, A.; von Meyer, A.; Ziegler, A.G. Reduced blood leukocyte and neutrophil numbers in the pathogenesis of type 1 diabetes. *Horm. Metab. Res.* **2013**, *45*, 467–470. [CrossRef] [PubMed]
18. Valle, A.; Giamporcaro, G.M.; Scavini, M.; Stabilini, A.; Grogan, P.; Bianconi, E.; Sebastiani, G.; Masini, M.; Maugeri, N.; Porretti, L.; et al. Reduction of circulating neutrophils precedes and accompanies type 1 diabetes. *Diabetes* **2013**, *62*, 2072–2077. [CrossRef] [PubMed]
19. Citro, A.; Valle, A.; Cantarelli, E.; Mercalli, A.; Pellegrini, S.; Liberati, D.; Daffonchio, L.; Kastsiuchenka, O.; Ruffini, P.A.; Battaglia, M.; et al. CXCR1/2 inhibition blocks and reverses type 1 diabetes in mice. *Diabetes* **2015**, *64*, 1329–1340. [CrossRef] [PubMed]
20. Haurogné, K.; Pavlovic, M.; Rogniaux, H.; Bach, J.M.; Lieubeau, B. Type 1 diabetes prone NOD mice have diminished Cxcr1 mRNA expression in polymorphonuclear neutrophils and CD4+ T lymphocytes. *PLoS ONE* **2015**, *10*, e0134365. [CrossRef] [PubMed]
21. Lappin, D.F.; Robertson, D.; Hodge, P.; Treagus, D.; Awang, R.A.; Ramage, G.; Nile, C.J. The influence of glycated hemoglobin on the cross susceptibility between type 1 diabetes mellitus and periodontal disease. *J. Periodontol.* **2015**, *86*, 1249–1259. [CrossRef] [PubMed]
22. Kashiwagi, Y.; Takedachi, M.; Mori, K.; Kubota, M.; Yamada, S.; Kitamura, M.; Murakami, S. High glucose-induced oxidative stress increases IL-8 production in human gingival epithelial cells. *Oral Dis.* **2016**, *22*, 578–584. [CrossRef] [PubMed]
23. Engebretson, S.P.; Vossughi, F.; Hey-Hadavi, J.; Emingil, G.; Grbic, J.T. The influence of diabetes on gingival crevicular fluid beta-glucuronidase and interleukin-8. *J. Clin. Periodontol.* **2006**, *33*, 784–790. [CrossRef] [PubMed]
24. Longo, P.L.; Artese, H.P.; Horliana, A.C.; Gomes, G.H.; Romito, G.A.; Dib, S.A.; Mayer, M.P. Inflammatory markers in gingival crevicular fluid of periodontitis patients with type 2 diabetes mellitus according to glycemic control: A pilot study. *Dent. Res. J. (Isfahan)* **2015**, *12*, 449–455. [CrossRef]
25. Mohamed, H.G.; Idris, S.B.; Ahmed, M.F.; Åstrøm, A.N.; Mustafa, K.; Ibrahim, S.O.; Mustafa, M. Influence of type 2 diabetes on local production of inflammatory molecules in adults with and without chronic periodontitis: A cross-sectional study. *BMC Oral Health* **2015**, *15*, 86. [CrossRef] [PubMed]
26. Purohit, S.; Sharma, A.; Hopkins, D.; Steed, L.; Bode, B.; Anderson, S.W.; Reed, J.C.; Steed, R.D.; Yang, T.; She, J.X. Large-scale discovery and validation studies demonstrate significant reductions in circulating levels of IL8, IL-1Ra, MCP-1, and MIP-1β in patients with type 1 diabetes. *J. Clin. Endocrinol. Metab.* **2015**, *100*, E1179–E1187. [CrossRef] [PubMed]
27. Hull, J.; Thomson, A.; Kwiatkowski, D. Association of respiratory syncytial virus bronchiolitis with the interleukin 8 gene region in UK families. *Thorax* **2000**, *55*, 1023–1027. [CrossRef] [PubMed]
28. Hu, N.; Westra, J.; Rutgers, A.; Doornbos-Van der Meer, B.; Huitema, M.G.; Stegeman, C.A.; Abdulahad, W.H.; Satchell, S.C.; Mathieson, P.W.; Heeringa, P.; et al. Decreased CXCR1 and CXCR2 expression on neutrophils in anti-neutrophil cytoplasmic autoantibody-associated vasculitides potentially increases neutrophil adhesion and impairs migration. *Arthritis Res. Ther.* **2011**, *13*, R201. [CrossRef] [PubMed]
29. Ryan, B.M.; Robles, A.I.; McClary, A.C.; Haznadar, M.; Bowman, E.D.; Pine, S.R.; Brown, D.; Khan, M.; Shiraishi, K.; Kohno, T.; et al. Identification of a functional SNP in the 3'UTR of CXCR2 that is associated with reduced risk of lung cancer. *Cancer Res.* **2015**, *75*, 566–575. [CrossRef] [PubMed]
30. Borilova Linhartova, P.; Vokurka, J.; Poskerova, H.; Fassmann, A.; Izakovicova Holla, L. Haplotype analysis of interleukin-8 gene polymorphisms in chronic and aggressive periodontitis. *Mediat. Inflamm.* **2013**, *2013*, 342351. [CrossRef] [PubMed]

31. Chapple, I.L.; Genco, R.; Working Group 2 of the Joint EFP/AAP Workshop. Diabetes and periodontal diseases: Consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases. *J. Periodontol.* **2013**, *84*, S106–S112. [CrossRef] [PubMed]
32. Borgnakke, W.S.; Ylöstalo, P.V.; Taylor, G.W.; Genco, R.J. Effect of periodontal disease on diabetes: Systematic review of epidemiologic observational evidence. *J. Periodontol.* **2013**, *84*, S135–S152. [CrossRef] [PubMed]
33. Iacopino, A.M. Periodontitis and diabetes interrelationships: Role of inflammation. *Ann. Periodontol.* **2001**, *6*, 125–137. [CrossRef] [PubMed]
34. Van Sickle, B.J.; Simmons, J.; Hall, R.; Raines, M.; Ness, K.; Spagnoli, A. Increased circulating IL-8 is associated with reduced IGF-1 and related to poor metabolic control in adolescents with type 1 diabetes mellitus. *Cytokine* **2009**, *48*, 290–294. [CrossRef] [PubMed]
35. Costa, K.L.; Taboza, Z.A.; Angelino, G.B.; Silveira, V.R.; Montenegro, R., Jr.; Haas, A.N.; Rego, R.O. Influence of periodontal disease on changes of glycated hemoglobin levels in type 2 diabetics: A retrospective cohort study. *J. Periodontol.* **2017**, *88*, 17–25. [CrossRef] [PubMed]
36. Polak, D.; Shapira, L. An update on the evidence for pathogenic mechanisms that may link periodontitis and diabetes. *J. Clin. Periodontol.* **2018**, *45*, 150–166. [CrossRef] [PubMed]
37. Podkówka, R.; Wiśniewska, J.; Korybalska, K.; Wieczorowska-Tobis, K.; Knapowski, J. Plasma level of interleukin-6 and interleukin-8 in the elderly. *Prz. Lek.* **2002**, *59*, 230–233. [PubMed]
38. Kim, C.S.; Park, H.S.; Kawada, T.; Kim, J.H.; Lim, D.; Hubbard, N.E.; Kwon, B.S.; Erickson, K.L.; Yu, R. Circulating levels of MCP-1 and IL-8 are elevated in human obese subjects and associated with obesity-related parameters. *Int. J. Obes. (Lond.)* **2006**, *30*, 347–1355. [CrossRef] [PubMed]
39. Li, G.; Yue, Y.; Tian, Y.; Li, J.L.; Wang, M.; Liang, H.; Liao, P.; Loo, W.T.; Cheung, M.N.; Chow, L.W. Association of matrix metalloproteinase (MMP)-1, 3, 9, interleukin (IL)-2, 8 and cyclooxygenase (COX)-2 gene polymorphisms with chronic periodontitis in a Chinese population. *Cytokine* **2012**, *60*, 552–560. [CrossRef] [PubMed]
40. Fredriksson, M.I. Effect of priming in subpopulations of peripheral neutrophils from patients with chronic periodontitis. *J. Periodontol.* **2012**, *83*, 1192–1199. [CrossRef] [PubMed]
41. Perlman, A.S.; Chevalier, J.M.; Wilkinson, P.; Liu, H.; Parker, T.; Levine, D.M.; Sloan, B.J.; Gong, A.; Sherman, R.; Farrell, F.X. Serum inflammatory and immune mediators are elevated in early stage diabetic nephropathy. *Ann. Clin. Lab. Sci.* **2015**, *45*, 256–263. [PubMed]
42. Lee, W.P.; Tai, D.I.; Lan, K.H.; Li, A.F.; Hsu, H.C.; Lin, E.J.; Lin, Y.P.; Sheu, M.L.; Li, C.P.; Chang, F.Y.; et al. The -251T allele of the interleukin-8 promoter is associated with increased risk of gastric carcinoma featuring diffuse-type histopathology in Chinese population. *Clin. Cancer Res.* **2005**, *11*, 6431–6441. [CrossRef] [PubMed]
43. Benakanakere, M.R.; Finoti, L.S.; Tanaka, U.; Grant, G.R.; Scarel-Caminaga, R.M.; Kinane, D.F. Investigation of the functional role of human Interleukin-8 gene haplotypes by CRISPR/Cas9 mediated genome editing. *Sci. Rep.* **2016**, *6*, 31180. [CrossRef] [PubMed]
44. Nibali, L.; Di Iorio, A.; Onabolu, O.; Lin, G.H. Periodontal infectogenomics: Systematic review of associations between host genetic variants and subgingival microbial detection. *J. Clin. Periodontol.* **2016**, *43*, 889–900. [CrossRef] [PubMed]
45. Mesia, R.; Gholami, F.; Huang, H.; Clare-Salzler, M.; Aukhil, I.; Wallet, S.M.; Shaddox, L.M. Systemic inflammatory responses in patients with type 2 diabetes with chronic periodontitis. *BMJ Open Diabetes Res. Care* **2016**, *4*, e000260. [CrossRef] [PubMed]
46. Ji, S.; Kim, Y.; Min, B.M.; Han, S.H.; Choi, Y. Innate immune responses of gingival epithelial cells to nonperiodontopathic and periodontopathic bacteria. *J. Periodontal. Res.* **2007**, *42*, 503–510. [CrossRef] [PubMed]
47. Kurgan, Ş.; Kansal, S.; Nguyen, D.; Stephens, D.; Koroneos, Y.; Hasturk, H.; Van Dyke, T.E.; Kantarci, A. Strain-specific impact of Fusobacterium nucleatum on neutrophil function. *J. Periodontol.* **2017**, *88*, 380–389. [CrossRef] [PubMed]
48. Bartova, J.; Borilova Linhartova, P.; Podzimek, S.; Janatova, T.; Svobodova, K.; Fassmann, A.; Duskova, J.; Belacek, J.; Izakovicova Holla, L. The effect of IL-4 gene polymorphisms on cytokine production in patients with chronic periodontitis and in healthy controls. *Mediat. Inflamm.* **2014**, *2014*, 85757. [CrossRef] [PubMed]
49. Armitage, G.C. Development of a classification system for periodontal diseases and conditions. *Ann. Periodontol.* **1999**, *4*, 1–6. [CrossRef] [PubMed]

50. Borilova Linhartova, P.; Bartova, J.; Poskerova, H.; Machal, J.; Vokurka, J.; Fassmann, A.; Izakovicova Holla, L. Apolipoprotein E gene polymorphisms in relation to chronic periodontitis, periodontopathic bacteria, and lipid levels. *Arch. Oral Biol.* **2015**, *60*, 456–462. [CrossRef] [PubMed]
51. Löe, H. The gingival index, the plaque index and the retention index systems. *J. Periodontol.* **1967**, *38*, 610–616. [CrossRef]
52. American Diabetes Association. Standards of Medical Care in Diabetes—2016. Classification and Diagnosis of Diabetes. *Diabetes Care* **2016**, *39*, S11–S22. [CrossRef]
53. Czech Diabetes Association. Diabetic Treatment. Glycated Hemoglobin and Diabetic Control. Available online: http://www.diabetickaasociace.cz/radi/co-na-vas-prozradi-glykovany-hemoglobin-o-kompenzaci-diabetu/ (accessed on 22 July 2017). (In Czech)
54. Sambrook, J.; Fritsch, E.F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring, NY, USA, 1989; pp. 9–17, ISBN 0-87969-309-6.

© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

4.3 Comment to the article ”Chemokine receptor 2 (*CXCR2*) gene variants and their association with periodontal bacteria in patients with chronic periodontitis”

Kavrikova D*, Borilova Linhartova P*, Lucanova S, Poskerova H, Fassmann A, Izakovicova Holla L. Chemokine receptor 2 (*CXCR2*) gene variants and their association with periodontal bacteria in patients with chronic periodontitis. Mediators Inflamm. 2019;2019:2061868. (IF 3.549; CELL BIOLOGY Q2, IMMUNOLOGY Q2)

*Authors contributed equally to this work.

Author contribution: P.B.L. designed the study, particularly drafted the paper, and carried out the genetic analysis.

Till now, two Brazilian studies have investigated the variability in *CXCR1* or *CXCR2* genes in CP patients; however, only the *CXCR2* genotypes and haplotypes have been associated with this oral disease (Viana et al., 2010; Scarel-Caminaga et al., 2011).

In relation to the previous study (Borilova Linhartova et al., 2018b, see chapter 4.2), the aim of this study was to investigate the *CXCR2* gene variability in CP patients and the non-periodontitis HC in a larger sample of the Czech population.

A total of 500 unrelated subjects participated in this case-control study: 329 CP patients and 171 non-periodontitis HC. The *CXCR2* rs2230054, *CXCR2* rs1126579, and *CXCR2* rs1126580 polymorphisms were determined using PCR techniques. Subgingival bacterial colonization was analyzed with a DNA microarray detection kit in an external laboratory (Stomagene, České Budějovice, Czech Republic).

No significant differences in allele, genotype, or haplotype frequencies of the *CXCR2* gene variants studied between patients with CP and the non-periodontitis HC were found ($P>0.05$). If we arrange haplotypes as genotypes, the carriers of the TCA/TTG (or TTG/TCA) variant seemed to be more susceptible to CP development ($P<0.05$). On the other hand, the number of carriers of this haplogenotype is too small to demonstrate any significant association with CP after correction for multiple comparisons. Viana et al. (2010) found that patients carrying the haplotypes TCA and CCG were more predetermined to CP development, whereas CCA and TCG haplotypes seemed to be protective against CP. Our result matches with the findings of the Brazilian study (Viana et al., 2010), where TTG/TCA and CTG/TCA haplotypes were associated with CP in two different populations.

Nevertheless, *A. actinomycetemcomitans* was detected more frequently in men who were positive for the *CXCR2* rs2230054 C allele and for the *CXCR2* rs1126579 T allele than in men with the other alleles (P<0.05, OR=2.31, 95%CI=1.03-5.20, and P<0.05, OR=2.54, 95%CI=1.13-5.71). In contrast, no statistically significant associations of CXCR2 variants with seven selected periodontal bacteria were found in women (P>0.05).

Although *A. actinomycetemcomitans* is more often associated with aggressive periodontitis than with CP, Gaetti-Jardim et al. (2008) detected the bacteria using the PCR method in 44 % of CP patients. What is more, the specific ILs variants or their combinations were associated with the presence of *A. actinomycetemcomitans* in CP patients (Bartova et al., 2014; Nibali et al., 2016; Cirelli et al., 2017).

Although none of the investigated SNPs in the *CXCR2* gene were associated with CP, the *CXCR2* gene variants can be associated with subgingival colonization of G^- bacteria in men with CP in the Czech population.

Hindawi
Mediators of Inflammation
Volume 2019, Article ID 2061868, 8 pages
https://doi.org/10.1155/2019/2061868

Research Article

Chemokine Receptor 2 (*CXCR2*) Gene Variants and Their Association with Periodontal Bacteria in Patients with Chronic Periodontitis

Denisa Kavrikova,[1] Petra Borilova Linhartova,[1,2] Svetlana Lucanova,[1] Hana Poskerova,[1] Antonin Fassmann,[1] and Lydie Izakovicova Holla[1,2]

[1]*Clinic of Stomatology, Institution Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Pekarska 664/53, 60200 Brno, Czech Republic*
[2]*Department of Pathophysiology, Faculty of Medicine, Masaryk University, Kamenice 5, 62500 Brno, Czech Republic*

Correspondence should be addressed to Lydie Izakovicova Holla; holla@med.muni.cz

Received 6 December 2018; Accepted 22 January 2019; Published 4 February 2019

Guest Editor: Nurcan Buduneli

Copyright © 2019 Denisa Kavrikova et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Periodontitis, an inflammatory disease caused by subgingival Gram-negative (G-) bacteria, is linked with loss of the connective tissue and destruction of the alveolar bone. In the regulation of inflammatory response, chemokine receptor 2 (CXCR2), a specific receptor for interleukin-8 and neutrophil chemoattractant, plays an important role. The first aim of this study was to investigate the *CXCR2* gene variability in chronic periodontitis (CP) patients and healthy nonperiodontitis controls in the Czech population. The second aim was to find a relation between *CXCR2* gene variants and the presence of periodontal bacteria. A total of 500 unrelated subjects participated in this case-control study. 329 CP patients and 171 healthy nonperiodontitis controls were analyzed using polymerase chain reaction techniques for three single-nucleotide polymorphisms (SNPs): +785C/T (rs2230054), +1208T/C (rs1126579), and +1440A/G (rs1126580). A DNA microarray detection kit was used for the investigation of the subgingival bacterial colonization, in a subgroup of CP subjects ($N = 162$). No significant differences in allele, genotype, haplotype, or haplogenotype frequencies of *CXCR2* gene variants between patients with CP and healthy controls ($P > 0.05$) were determined. Nevertheless, *Aggregatibacter actinomycetemcomitans* was detected more frequently in men positive for the C allele of the *CXCR2* +785C/T polymorphism (61.8% vs. 41.1%, $P < 0.05$; OR = 2.31, 95% CI = 1.03-5.20) and for the T allele of the *CXCR2* +1208C/T variant (61.8% vs. 38.9%, $P < 0.05$; OR = 2.54, 95% CI = 1.13-5.71). In contrast, no statistically significant associations of *CXCR2* variants with seven selected periodontal bacteria were found in women. Although none of the investigated SNPs in the *CXCR2* gene was associated with CP, the *CXCR2* gene variants can be associated with subgingival colonization of G- bacteria in men with CP in the Czech population.

1. Introduction

Periodontitis is a multifactorial disease that is primarily caused by specific pathogen-associated molecular patterns (PAMPs) and bacterial virulence factors; they trigger an inflammatory host response which results in periodontal tissue destruction and loss of teeth [1, 2]. Chronic periodontitis (CP), the most common form of periodontitis in adults, is either localized or generalized, based on the number of affected sites. The destruction corresponds to the presence of local factors, with a slow-to-moderate rate of progression, but may have periods of rapid progression [3]. CP is strongly associated with "red complex" Gram-negative (G-) bacteria, including *Tannerella forsythia*, *Porphyromonas gingivalis*, and *Treponema denticola* [1, 4]. Although *Aggregatibacter actinomycetemcomitans* is supposed to be the main etiological

agent of the aggressive form of periodontitis [5], this bacterium is also connected with CP and some nonoral infections [6].

The host response to anaerobic G- bacteria and their products is an important determinant for progression of periodontal disease. There are a few major risk factors, such as genetic predispositions, systemic diseases, or smoking, which affect the microbial composition in the oral cavity [7, 8]. Cytokines, mediators of host defense and also of periodontal tissue destruction, are considered to be important molecules in the etiopathogenesis of periodontal diseases [9].

Interleukin-8 (IL-8, CXCL8) is known as neutrophil-activating protein-1 (NAP-1) [10, 11]. The effect of IL-8 is mediated by its two receptors—7 transmembrane class A (rhodopsin-like) G protein-coupled receptors (7-TM-GPCRs), so called CXCR1 and CXCR2 [12, 13]. CXCR1 and CXCR2 are expressed on a wide range of leukocytes, including neutrophils, mast cells, and also oral epithelial cells [14, 15]. They are involved in the multiple biological activities, such as initiation and amplification of acute inflammatory reaction, as well as tumor growth, angiogenesis, and metastasis [16–18]. Experimental data suggest that IL-8 and its receptors participate in the elimination of pathogens [19]. A study by Zenobia et al. shows that the recruitment of neutrophils to gingival tissue does not require commensal bacterial colonization but is entirely dependent on *CXCR2* expression [20].

Only a few studies have investigated the variability in *CXCR1* or *CXCR2* genes in relation to CP [21–23], especially in the Brazilian population; however, only the *CXCR2* genotypes and haplotypes have been associated with CP [21]. Based on our previous investigation of *IL-8* gene variability and its association with periodontal bacteria in patients with CP [24], we assumed the role of IL-8 receptor in the etiopathogenesis of periodontal disease.

The first aim of our study was to analyze three SNPs in the *CXCR2* gene +785C/T (rs2230054), +1208T/C (rs1126579), and +1440G/A (rs1126580) in CP patients and healthy nonperiodontitis controls in the Czech population; the second aim was to associate these SNPs with the presence of seven periodontal bacteria in subjects with CP.

2. Materials and Methods

2.1. Subjects. This case-control association study comprised 500 unrelated Caucasian subjects of exclusively Czech ethnicity from the South Moravian Region. Subjects with CP (number of subjects, $N = 329$) were recruited from the Periodontology Department, Clinic of Stomatology, St. Anne's Faculty Hospital, Brno, over the period of 2013–2018. Healthy nonperiodontitis controls ($N = 171$) were selected from patients who had been referred to the Clinic of Stomatology for reasons other than periodontal disease (such as preventive dental check-ups, dental decay, and orthodontic consultations) during the same period as CP patients, and they were of similar age, gender, and smoking status. Similarly, like the patients, all controls were in good systemic health and had minimally 20 remaining teeth. The exclusion criteria included the presence of diabetes mellitus, cardiovascular disorders (such as hypertension or coronary artery diseases), immunodeficiency, current pregnancy or lactation, malignant diseases, immunosuppression due to medication or concurrent illness, the use of anti-inflammatory drugs or antibiotics during a six-week recruitment period, and the inability to consent [25].

Table 1: Demographic data of CP patients and healthy nonperiodontitis controls.

Characteristics	Controls ($N = 171$)	CP ($N = 329$)
Age (mean ± SD, years)	47.56 ± 11.80	54.03 ± 8.99*
Gender (males/females)	82/89	150/179
Smoking (no/yes) (%)	73.68/26.32	72.49/27.18
BMI (mean ± SD, kgm^{-2})	25.45 ± 3.61	26.20 ± 3.77
PD (mean ± SD, mm)	1.21 ± 0.24	3.26 ± 0.81*
AL (mean ± SD, mm)	1.33 ± 0.21	3.94 ± 1.05*
PI (mean ± SD, mm)	0.34 ± 0.14	0.83 ± 0.49*
GI (mean ± SD, mm)	0.38 ± 0.31	0.81 ± 0.36*

AL = attachment loss; CP = chronic periodontitis; GI = gingival index; N = number of subjects; PD = probing depth; PI = plaque index; SD = standard deviation. *$P < 0.05$.

Clinical diagnosis of nonperiodontitis/periodontitis was based on a thorough examination (PI = plaque index, GI = gingival index, etc.), medical/dental history, tooth mobility, and radiographic evaluation. Probing depth (PD) and attachment loss (AL) were collected with a UNC-15 periodontal probe from six sites on every tooth present. The loss of the alveolar bone was determined radiographically, and the decrease in alveolar bone levels was assessed with the Mühlemann index [24]. All participants, no matter whether they agreed or declined to participate or were excluded from the study, were offered periodontitis treatment. The patients were firstly examined by a periodontist, and they had not received scaling and/or root planing minimally six months before measuring periodontal indices [25].

According to their smoking history, the subjects were split into the following groups: nonsmokers (subjects who never smoked) and smokers (former smokers for ≥5 pack-years or current smokers). The pack-years were calculated by multiplying the number of years of smoking by the average number of cigarette packs smoked per day [23]. The demographic data of the studied subjects are shown in Table 1.

2.2. Genetic Analysis. Genomic DNA was isolated from peripheral blood by a standard protocol. It was archived in the DNA bank at the Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic. Three SNPs in the *CXCR2* gene (+785C/T (rs2230054), +1208C/T (rs1126579), and +1440A/G (rs1126580)) were analyzed.

For detection of SNP in the *CXCR2* gene at position +785C/T (rs2230054), the original restriction fragment length polymorphism (RFLP-PCR) method with mismatch primers was introduced. Primers were designed by the

Primer3Output program. PCR was carried out in a volume of 25 μL containing 100 ng of genomic DNA, 0.5 μM of each primer (Fwd: 5′-TCGTCCTCATCTTCCCGCT and Rev: 5′-GGAGTCCATGGCGAAACTTC), 4 U of *Taq* DNA polymerase (Thermo Scientific, Waltham, USA), 2 mM of $MgCl_2$, $MgCl_2$-free reaction buffer with NH_4SO_3 (Thermo Scientific, Waltham, USA), and 0.5 mM deoxyribonucleoside triphosphate mix (Thermo Scientific, Waltham, USA). Denaturation for 5 min at 95°C was followed by 35 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min. The last synthesis step was extended to 7 min at 72°C. The restriction of the PCR product (210 bp) was performed in a volume of 25 μL consisting of 15 μL of the PCR product, 10x CutSmart Buffer, and 4 U of *BsrBI* enzyme (New England Biolabs, Hitchin, United Kingdom), and incubation was done overnight at 37°C. The length of products after restriction digestion was 210 bp for the TT genotype, 210 bp + 193 bp + 17 bp for CT, and 193 bp + 17 bp for CC. The fragments were visualized by 3.0% agarose gel electrophoresis by ethidium bromide. Sizing of the product was performed using a GeneRuler™ 50 bp DNA Ladder (Thermo Scientific, Waltham, USA).

SNP +1208C/T (rs1126579) in the *CXCR2* gene was genotyped using the 5′ nuclease TaqMan® assay C_8841198_10 for allelic discrimination according to the manufacturer's instructions (Life Technologies, Grand Island, NY, USA). Allele genotyping from fluorescence measurements was then obtained using the ABI PRISM 7000 Sequence Detection System. SDS version 1.2.3 software was used to analyze real-time and endpoint fluorescence data.

The +1440A/G (rs1126580) SNP was genotyped by allele-specific PCR analysis according to the previously published method [26], with a slight modification. A set of appropriate sequences of allele-specific primers and control primers was used: for the allele-specific DNA fragment (Fwd: 5′-AGGCTGGCCAACGGGG/A and Rev: 5′-TCAT AGCAGCTTATTCACAAGAC) and for the control DNA fragment (Fwd: 5′-TGCCAAGTGGAGCACCCAA and Rev: 5′-GCATCTTGCTCTGTGCAGAT). There is a difference between the sequences of the allele-specific primers used in our study and those in the work of Renzoni et al. [26]. The length of amplified DNA fragments was also different. The presence of an allele-specific band (435 bp) of the expected size in conjunction with a control band (796 bp) was considered to be positive evidence for each particular allele. The absence of an allele-specific band and the presence of a control band were considered to be a negative indication for a particular allele. Briefly, PCR was carried out in a volume of 25 μL containing 100 ng of genomic DNA, 0.5 μM of each allele-specific primer, 0.4 μM of each control primer, 2.5 U of *Taq* DNA polymerase (Thermo Scientific, Waltham, USA), 2 mM of $MgCl_2$, $MgCl_2$-free reaction buffer with NH_4SO_3 (Thermo Scientific, Waltham, USA), and 0.5 mM deoxyribonucleoside triphosphate mix (Thermo Scientific, Waltham, USA). Denaturation for 5 min at 95°C was followed by 35 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min. The last synthesis step was extended to 7 min at 72°C. The fragments were visualized by 2.0% agarose gel electrophoresis by ethidium bromide. Sizing of the product was performed using a GeneRuler™ 50 bp DNA Ladder (Thermo Scientific, Waltham, USA).

2.3. Microbial Analysis. The analyses of seven selected periodontal bacteria based on a DNA microarray detection kit (Protean Ltd., Ceske Budejovice, Czech Republic) have been described previously [24, 27]. The presence of bacterial colonization (*A. actinomycetemcomitans*, *T. forsythia*, *P. gingivalis*, *T. denticola*, *Parvimonas micra*, *Prevotella intermedia*, and *Fusobacterium nucleatum*) in subgingival pockets was examined in a subgroup of 162 CP patients before subgingival scaling. Bacterial load was assessed semiquantitatively: (-) undetected, corresponding to a number of bacteria less than 10^3; (+) slightly positive, which corresponds to a number of bacteria from 10^3 to 10^4; (++) positive, corresponding to a number of bacteria from 10^4 to 10^5; and (+++) strongly positive, corresponding to a number of bacteria exceeding 10^5 [28]. The diagnosis of the specific bacterial infection was considered positive when the number of bacterial cells surpassed 10^3 [23].

2.4. Statistical Analysis. Standard descriptive statistics were applied: mean with standard deviations (SD) or median with quartiles for quantitative variables and absolute and relative frequencies for categorical variables. One-way analysis of variance (ANOVA) or Kruskal-Wallis ANOVA was performed to compare continuous variables among the groups. The allele frequencies were calculated from the observed numbers of genotypes. The differences in the allele frequencies were compared by the Fisher exact test, and genotype/haplogenotype frequencies and Hardy-Weinberg equilibrium (HWE) were tested by the χ^2 test. To examine the linkage disequilibrium (LD) between polymorphisms, pairwise LD coefficients (D') and haplotype frequencies were calculated using the SNP Analyzer 2 program (http://snp.istech.info/istech/board/login_form.jsp). Odds ratio (OR), confidence intervals (CI), and P values were calculated. P values less than 0.05 were considered statistically significant. Where appropriate, the Bonferroni correction was used to adjust the level according to the number of independent comparisons to the overall value of 0.05. The adjusted P values are denoted as P_{corr}. Power analysis was performed with respect to the case-control design of the study, taking the incidence rate of markers. Statistical analysis was performed using the statistical package Statistica v. 13 (StatSoft Inc., USA).

3. Results

3.1. Case-Control Study. Our population sample consisted of 232 males and 268 females (CP patients 45.6%/54.4%, controls 48.0%/52.0%). 26.3% of CP patients and similarly 27.2% of healthy nonperiodontitis controls were smokers ($P > 0.05$). No significant differences in means of the body mass index (BMI) between CP patients and controls ($P > 0.05$, mean ± SD: 25.45 ± 3.61 kg m^{-2} vs. 26.20 ± 3.77 kg m^{-2}, respectively) were detected. Groups of cases and controls were different according to PD, AL, PI, and GI ($P < 0.01$); in CP patients, all mean values were higher

Table 2: *CXCR2* genotype and allele frequencies in CP patients and healthy nonperiodontitis controls.

Genotypes Alleles	Controls $N = 171$ (%)	CP $N = 329$ (%)	P value	OR (95% CI)
CXCR2 +785				
CC	41 (24.0)	81 (24.6)	—	1.00
CT	93 (54.4)	166 (50.5)	0.37	0.90 (0.57-1.42)
TT	37 (21.6)	82 (24.9)	0.39	1.12 (0.65-1.93)
C allele	175 (51.2)	328 (49.8)	—	1.00
T allele	167 (48.8)	330 (50.2)	0.37	1.05 (0.81-1.37)
CXCR2 +1208				
CC	45 (26.3)	91 (27.7)	—	1.00
CT	90 (52.6)	174 (52.9)	0.47	0.96 (0.62-1.48)
TT	36 (21.1)	64 (19.5)	0.37	0.88 (0.51-1.51)
C allele	180 (52.6)	356 (54.1)	—	1.00
T allele	162 (47.4)	302 (45.9)	0.35	0.94 (0.73-1.22)
CXCR2 +1440				
AA	35 (20.5)	59 (17.9)	—	1.00
AG	89 (52.0)	171 (52.0)	0.34	1.14 (0.70-1.86)
GG	47 (27.5)	99 (30.1)	0.25	1.25 (0.73-2.15)
A allele	159 (46.5)	289 (43.9)	—	1.00
G allele	183 (53.5)	369 (56.1)	0.24	1.11 (0.85-1.44)

CI = confidential interval; CP = chronic periodontitis; N = number of subjects; OR = odds ratio.

than those in controls. The demographic data of the studied subjects are given in Table 1.

The sample size of the study was optimized to reach relevant detectable effect size keeping the standard level of statistical errors, i.e., type I error 0.05 and type II error 0.20 or power of the test 0.80, respectively. The power calculation was optimized on the basis of the Fisher exact and goodness-of-fit tests as statistical tools used in comparing the principal endpoints in the study. Regarding the background relative frequency of the examined phenomenon as 50%, the reached sample size (171 controls, 329 cases, control : case ratio approx. 0.5) enabled to distinguish the difference in relative distribution of any entity (±13%) as statistically significant. Similarly, regarding the mean relative frequency as 50%, the study is able to detect difference (±13%) with 95% confidence.

3.2. SNPs and Haplotype Analysis. The studied polymorphisms +785C/T (rs2230054), +1208C/T (rs1126579), and +1440A/G (rs1126580) were in HWE in the control group ($P > 0.05$). No significant differences of all allele and genotype frequencies between the CP and control groups were found (see Table 2).

The distribution of genotype frequencies of all studied *CXCR2* gene variants was similar between men and women (data not shown). The SNPs in the *CXCR2* gene (+785C/T (rs2230054), +1208C/T (rs1126579), and +1440A/G (rs1126580)) were in very tight LD with each other to various degrees ($|D'| = 0.72 - 0.94$). Only the haplogenotype TCA/TTG was found more frequently in CP patients than in controls (0.0% vs. 2.4%, $P < 0.05$, $P_{corr} > 0.05$), but the number of subjects in both groups was very low. In our population, no other association between *CXCR2* haplotypes or haplogenotypes and CP was found ($P > 0.05$ for both, see Tables 3 and 4, respectively).

3.3. Microbial Analysis. There were no relationships between variability in the three studied *CXCR2* SNPs and the presence of seven periodontal bacteria in 162 CP patients, and only *A. actinomycetemcomitans* was marginally associated with *CXCR2* +785C/T SNP ($P = 0.06$). *A. actinomycetemcomitans* occurred more frequently in men positive for the C allele of the *CXCR2* +785C/T polymorphism (61.8% vs. 41.1%, $P < 0.05$; OR = 2.31, 95% CI = 1.03-5.20) and for the T allele of the *CXCR2* +1208C/T variant (61.8% vs. 38.9%, $P < 0.05$; OR = 2.54, 95% CI = 1.13-5.71). The presence of *P. micra* was marginally associated with the T allele of +1208 SNP for the group of male CP patients (49.0% vs. 27.3%, $P = 0.05$; OR = 2.56, 95% CI = 0.93-7.08; see Table 5). In contrast, there were no differences between the frequencies of *CXCR2* gene variants and the presence of periodontal bacteria in the group of CP women ($P > 0.05$, data not shown).

4. Discussion

Periodontal disease is characterized by inflammatory processes of tissues surrounding the teeth in response to bacterial stimulation. This inflammatory process is responsible for the progressive loss of the collagen attachment of the tooth to the alveolar bone, leading to bone loss [29]. According to the statistical report by the Ministry of Health of the Czech Republic, 15-20% of the Czech population aged 35-44 years suffered from periodontal disease in 2014 [30].

Table 3: Estimated frequencies (%) of *CXCR2* haplotypes in CP patients and healthy nonperiodontitis controls.

CXCR2 +785C/T	*CXCR2* +1208C/T	*CXCR2* +1440A/G	Controls (N = 171)	CP (N = 329)	P value	OR (95% CI)
C	T	G	42.3	41.4	0.79	0.96 (0.74-1.26)
T	C	A	39.6	37.9	0.62	0.94 (0.72-1.22)
T	C	G	5.8	8.3	0.14	1.49 (0.87-2.56)
C	C	A	4.7	5.3	0.73	1.11 (0.60-2.09)
T	T	G	2.8	3.7	0.53	1.28 (0.58-2.81)
C	C	G	2.5	2.6	0.92	1.04 (0.46-2.34)
C	T	A	1.6	0.6	0.10	0.31 (0.07-1.30)
T	T	A	0.6	0.3	0.52	0.52 (0.07-3.70)

CI = confidential interval; CP = chronic periodontitis; N = number of subjects; OR = odds ratio.

Table 4: Distribution of *CXCR2* haplotypes (arranged as genotypes) in CP patients and healthy nonperiodontitis controls.

Haplogenotypes +785 +1208 +1440/+785 +1208 +1440	Controls N = 171 (%)	CP N = 329 (%)	P value	OR (95% CI)
CTG/CTG	27 (15.8)	52 (15.8)	0.55	1.00 (0.60-1.66)
CTG/TCA	67 (39.2)	128 (38.6)	0.51	0.99 (0.68-1.44)
CTG/TCG	6 (3.5)	12 (3.6)	0.58	1.04 (0.38-2.82)
CTG/CCA	5 (2.9)	11 (3.3)	0.52	1.15 (0.39-3.36)
CTG/TTG	6 (3.5)	5 (1.5)	0.13	0.42 (0.13-1.41)
CTG/CCG	6 (3.5)	11 (3.3)	0.55	0.95 (0.35-2.62)
CTG/CTA	2 (1.2)	2 (0.6)	0.42	0.52 (0.07-3.70)
CTG/TTA	0 (0.0)	2 (0.6)	0.43	#
TCA/TCA	21 (12.3)	42 (12.8)	0.50	1.05 (0.60-1.83)
TCA/TCG	12 (7.0)	21 (6.4)	0.46	0.90 (0.43-1.88)
TCA/CCA	9 (5.3)	13 (4.0)	0.32	0.74 (0.31-1.77)
TCA/TTG	0 (0.0)	8 (2.4)	0.03*	#
TCA/CCG	3 (1.8)	5 (1.5)	0.55	0.86 (0.20-3.66)
TCA/CTA	2 (1.2)	0 (0.0)	0.12	#
TCA/TTA	2 (1.2)	0 (0.0)	0.12	#
TCG/TCG	0 (0.0)	5 (1.5)	0.12	#
TCG/TTG	1 (0.6)	3 (0.9)	0.58	1.56 (0.16-15.15)
TCG/CCG	0 (0.0)	1 (0.3)	0.66	#
CCA/CCA	0 (0.0)	3 (0.9)	0.28	#
CCA/CCG	0 (0.0)	1 (0.3)	0.95	#
CCA/CTA	1 (0.6)	1 (0.3)	0.57	0.52 (0.03-8.34)
TTG/TTG	1 (0.6)	3 (0.9)	0.58	1.56 (0.16-15.15)

CI = confidential interval; CP = chronic periodontitis; N = number of subjects; OR = odds ratio. *$P < 0.05$ by the Fisher exact test (without correction for multiple comparisons), but there is a low N in both groups. #OR not calculated because of the presence of zero.

Chemokines and their receptors play important roles in immunological responses, and thus their genetic contribution to various human inflammatory disorders needs investigation [31]. Several reports have suggested that the *CXCR2* variants might influence the susceptibility to chronic inflammatory conditions, especially rheumatoid and respiratory diseases [32–35]. The *CXCR2* gene variability has been associated with several disorders like systemic sclerosis and cryptogenic obstructive pulmonary disease [32]. In Slovakia, children with the SNP +1208T allele were significantly unrepresented in the recurrent acute pyelonephritis subgroup, and the carriage of the T allele (TT+CT genotypes vs. CC genotype) was linked with a reduced risk of developing this disease [36]. Moreover, analysis of SNP +1208 with serum levels of IL-8, its endogenous ligand, supports an interaction whereby the variant +1208T allele and high serum IL-8 confer syner-

Table 5: *CXCR2* gene variants and the presence of periodontal bacteria in 62 male CP patients.

Allele frequencies (%)		*CXCR2* +785		*CXCR2* +1208		*CXCR2* +1440	
		C	T	C	T	A	G
A. actinomycetemcomitans	Neg. $N = 90$	41.1	58.9	61.1	38.9	45.6	54.4
	Pos. $N = 34$	61.8*	38.2	38.2	61.8*	29.4	70.6
T. forsythia	Neg. $N = 14$	35.7	64.3	50.0	50.0	35.7	64.3
	Pos. $N = 110$	48.2	51.8	55.5	44.5	41.8	58.2
P. gingivalis	Neg. $N = 38$	47.4	52.6	55.3	44.7	36.8	63.2
	Pos. $N = 86$	46.5	53.5	54.7	45.3	43.0	57.0
T. denticola	Neg. $N = 40$	52.5	47.5	47.5	52.5	40.0	60.0
	Pos. $N = 84$	44.0	56.0	58.3	41.7	41.7	58.3
P. micra	Neg. $N = 22$	31.8	68.2	72.7	27.3	40.9	59.1
	Pos. $N = 102$	50.0	50.0	51.0	49.0*	41.2	58.8
P. intermedia	Neg. $N = 56$	50.0	50.0	53.6	46.4	35.7	64.3
	Pos. $N = 68$	44.1	55.9	55.9	44.1	45.6	54.4
F. nucleatum	Neg. $N = 2$	0.0	100.0	100.0	0.0	0.0	100.0
	Pos. $N = 122$	47.5	52.5	54.1	45.9	41.8	58.2

N = number of alleles; Neg. = negative; Pos. = positive. * $P \leq 0.05$.

were previously investigated in Brazilian patients with CP [21]. We detected similar allele, genotype, and haplotype frequencies of the all studied *CXCR2* gene polymorphisms between CP patients and controls ($P > 0.05$). In contrast, the +1440GG genotype, originally described by Viana et al. [37], was suggested as a protective factor against CP in Brazilians [21]. The differences between the results in the Czech and Brazilian study [21] could be caused by the interpopulation variability. The *CXCR2* +1440 minor allele frequencies were found to be 46.5% in Czech healthy nonperiodontitis controls vs. 57.4% in Brazilian controls [21]. However, our result is in line with the minor allele frequency (43.3%) in the European population [38].

If we arranged haplotypes as genotypes, the carriers of the TCA/TTG (or TTG/TCA) variant seemed to be more susceptible to CP development ($P < 0.05$). On the other hand, the number of carriers of this haplogenotype is too small to demonstrate any significant association with CP after correction for multiple comparisons. Viana et al. found that patients carrying the haplotypes TCA and CCG were more predetermined to CP development, whereas CCA and TCG haplotypes seemed to be protective against CP. In addition, white nonsmoking patients carrying the CTG/TCA variant were more likely to develop periodontal disease, whereas CTG/TCG patients seemed to be protected [21]. Our result matches with a comparable finding elsewhere: TTG/TCA and CTG/TCA haplotypes were associated with CP risk in two different populations, i.e., the Czech and Brazilian [21]. In addition, no CTG/TCG haplotype carrier was present in our population.

A lot of studies have focused on the relationship between the selected gene variants and the presence of periodontal bacteria studied in the recent review and meta-analysis by Nibali et al. [39]. Evidence suggests that genetic factors can influence periodontitis risk, modulating disease elements such as the susceptibility to microbial colonization and the nature of subsequent host-microbe interaction [40, 41]. Our previous research into *IL-8* gene polymorphisms in CP and aggressive periodontitis (AgP) patients shows the association between *IL-8* genotypes and the occurrence of specific periodontal bacteria [24]. In our earlier study, we reported significant differences in the colonization of the oral cavity with *P. gingivalis* (70.5% in CP patients vs. 28% in controls), *T. forsythia* (92.3% in CP patients vs. 56.3% in controls), and *P. micra* (87.2% in CP patients vs. 56.3% in controls) between CP patients and healthy controls [28]. We also determined that *IL-17A* -197A/G (rs2275913) polymorphism was associated with the presence of *T. forsythia* and *T. denticola* in CP patients [27] and that *IL-4* gene polymorphisms in CP patients could predispose to altered cytokine production after bacterial stimulation [42].

Although *A. actinomycetemcomitans* is more often associated with AgP than with CP, Gaetti-Jardim et al. [43] detected the bacteria by the PCR method in 44% of CP patients. In the Brazilian study, the *IL-4* haplotypes, but not the *IL-8* haplotypes, were associated with the presence of *A. actinomycetemcomitans* in CP patients [41]. Nibali et al. reported an association between the variability in the *IL-6* gene and *A. actinomycetemcomitans* in CP patients. The strong association of *IL-6* -174 GG homozygotes with the presence of *A. actinomycetemcomitans* in all subjects and in the subgroup of only white subjects was observed [42]. In our preliminary study, no significant association between the *CXCR2* +1208C/T (rs1126579) SNP and IL-8 plasma levels and the occurrence of the selected periodontal bacteria in 41 CP patients was found [24]. This result was confirmed in the present study on a larger sample size ($N = 162$). After the gender stratification, the presence of *A. actinomycetemcomitans* was significantly associated with *CXCR2* +785C/T and *CXCR2* +1208C/T SNPs, but only in Czech men.

5. Conclusions

This study did not confirm any significant association between the investigated SNPs in the *CXCR2* gene and chronic periodontitis. However, the *CXCR2* gene variants can be associated with subgingival colonization of the selected G- bacteria in men with CP in the Czech population.

Data Availability

The clinical and genetic data used to support the findings of this study are restricted by the Committees for Ethics of the Faculty of Medicine, Masaryk University, Brno (no. 13/2013), in order to protect patient privacy. Data are available from Lydie Izakovicova Holla (holla@med.muni.cz) for researchers who meet the criteria for access to confidential data.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

D.K. and P.B.L. are responsible for the conceptualization, methodology, validation, formal analysis, investigation, data curation, visualization, and writing of the manuscript (original draft preparation), S.L., H.P., and A.F. are responsible for the methodology and writing of the manuscript (review and editing), and L.I.H is responsible for the conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing of the manuscript (review and editing), and supervision. D.K. and P.B.L. contributed equally to this work.

Acknowledgments

This study was supported by a grant from the Czech Science Foundation (GB14-37368G), the project of Grant Agency of Masaryk University (MUNI/A/1008/2017), and funds granted by the Faculty of Medicine, MU, to junior researcher Petra Borilova Linhartova.

References

[1] S. S. Socransky, A. D. Haffajee, M. A. Cugini, C. Smith, and R. L. J. Kent, "Microbial complexes in subgingival plaque," *Journal of Clinical Periodontology*, vol. 25, no. 2, pp. 134–144, 1998.

[2] G. C. Armitage, "Comparison of the microbiological features of chronic and aggressive periodontitis," *Periodontology*, vol. 53, no. 1, pp. 70–88, 2010.

[3] J. Highfield, "Diagnosis and classification of periodontal disease," *Australian Dental Journal*, vol. 54, Suppl 1, pp. S11–S26, 2009.

[4] S. Tomita, S. Kasai, Y. Ihara et al., "Effects of systemic administration of sitafloxacin on subgingival microflora and antimicrobial susceptibility profile in acute periodontal lesions," *Microbial Pathogenesis*, vol. 71-72, pp. 1–7, 2014.

[5] C. H. Åberg, P. Kelk, and A. Johansson, "*Aggregatibacter actinomycetemcomitans*: virulence of its leukotoxin and association with aggressive periodontitis," *Virulence*, vol. 6, no. 3, pp. 188–195, 2015.

[6] B. Henderson, J. M. Ward, and D. Ready, "*Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*: a triple A* periodontopathogen?," *Periodontology 2000*, vol. 54, no. 1, pp. 78–105, 2010.

[7] P. Sudhakara, A. Gupta, A. Bhardwaj, and A. Wilson, "Oral dysbiotic communities and their implications in systemic diseases," *Dentistry Journal*, vol. 6, no. 2, p. 10, 2018.

[8] J. L. Ebersole, M. J. Steffen, M. V. Thomas, and M. Al-Sabbagh, "Smoking-related cotinine levels and host responses in chronic periodontitis," *Journal of Periodontal Research*, vol. 49, no. 5, pp. 642–651, 2014.

[9] A. Yoshimura, Y. Hara, T. Kaneko, and T. Kato, "Secretion of IL-1β, TNF-α, IL-8 and IL-1ra by human polymorphonuclear leukocytes in response to lipopolysaccharides from periodontopathic bacteria," *Journal of Periodontal Research*, vol. 32, no. 3, pp. 279–286, 1997.

[10] P. M. Murphy and H. L. Tiffany, "Cloning of complementary DNA encoding a functional human interleukin-8 receptor," *Science*, vol. 253, no. 5025, pp. 1280–1283, 1991.

[11] H. Sprenger, A. R. Lloyd, L. L. Lautens, T. I. Bonner, and D. J. Kelvin, "Structure, genomic organization, and expression of the human interleukin-8 receptor B gene," *The Journal of Biological Chemistry*, vol. 269, no. 15, pp. 11065–11072, 1994.

[12] M. S. D. Kormann, A. Hector, V. Marcos et al., "CXCR1 and CXCR2 haplotypes synergistically modulate cystic fibrosis lung disease," *The European Respiratory Journal*, vol. 39, no. 6, pp. 1385–1390, 2012.

[13] M. Kaur and D. Singh, "Neutrophil chemotaxis caused by chronic obstructive pulmonary disease alveolar macrophages: the role of CXCL8 and the receptors CXCR1/CXCR2," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 347, no. 1, pp. 173–180, 2013.

[14] M. Juremalm and G. Nilsson, "Chemokine receptor expression by mast cells," *Chemical Immunology and Allergy*, vol. 87, pp. 130–144, 2005.

[15] S. A. Khurram, L. Bingle, B. M. McCabe, P. M. Farthing, and S. A. Whawell, "The chemokine receptors CXCR1 and CXCR2 regulate oral cancer cell behaviour," *Journal of Oral Pathology & Medicine*, vol. 43, no. 9, pp. 667–674, 2014.

[16] S. K. Raghuwanshi, Y. Su, V. Singh, K. Haynes, A. Richmond, and R. M. Richardson, "The chemokine receptors CXCR1 and CXCR2 couple to distinct G protein-coupled receptor kinases to mediate and regulate leukocyte functions," *The Journal of Immunology*, vol. 189, no. 6, pp. 2824–2832, 2012.

[17] M. L. Varney, S. Singh, A. Li, R. Mayer-Ezell, R. Bond, and R. K. Singh, "Small molecule antagonists for CXCR2 and CXCR1 inhibit human colon cancer liver metastases," *Cancer Letters*, vol. 300, no. 2, pp. 180–188, 2011.

[18] S. Singh, S. Wu, M. Varney, A. P. Singh, and R. K. Singh, "CXCR1 and CXCR2 silencing modulates CXCL8-dependent endothelial cell proliferation, migration and capillary-like structure formation," *Microvascular Research*, vol. 82, no. 3, pp. 318–325, 2011.

[19] R. C. Russo, C. C. Garcia, M. M. Teixeira, and F. A. Amaral, "The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases," *Expert Review of Clinical Immunology*, vol. 10, no. 5, pp. 593–619, 2014.

[20] C. Zenobia, X. L. Luo, A. Hashim et al., “Commensal bacteria-dependent select expression of CXCL2 contributes to periodontal tissue homeostasis,” *Cellular Microbiology*, vol. 15, no. 8, pp. 1419–1426, 2013.

[21] A. C. Viana, Y. J. Kim, K. M. C. Curtis et al., “Association of haplotypes in the CXCR2 gene with periodontitis in a Brazilian population,” *DNA and Cell Biology*, vol. 29, no. 4, pp. 191–200, 2010.

[22] R. M. Scarel-Caminaga, K. M. C. Curtis, R. Renzi et al., “Variation in the CXCR1 gene (IL8RA) is not associated with susceptibility to chronic periodontitis,” *Journal of Negative Results in Biomedicine*, vol. 10, no. 1, p. 14, 2011.

[23] P. Borilova Linhartova, D. Kavrikova, M. Tomandlova et al., “Differences in interleukin-8 plasma levels between diabetic patients and healthy individuals independently on their periodontal status,” *International Journal of Molecular Sciences*, vol. 19, no. 10, article 3214, 2018.

[24] P. Borilova Linhartova, J. Vokurka, H. Poskerova, A. Fassmann, and L. Izakovicova Holla, “Haplotype analysis of interleukin-8 gene polymorphisms in chronic and aggressive periodontitis,” *Mediators of Inflammation*, vol. 2013, Article ID 342351, 8 pages, 2013.

[25] P. Borilova Linhartova, J. Bartova, H. Poskerova et al., “Apolipoprotein E gene polymorphisms in relation to chronic periodontitis, periodontopathic bacteria, and lipid levels,” *Archives of Oral Biology*, vol. 60, no. 3, pp. 456–462, 2015.

[26] E. Renzoni, P. Lympany, P. Sestini et al., “Distribution of novel polymorphisms of the interleukin-8 and CXC receptor 1 and 2 genes in systemic sclerosis and cryptogenic fibrosing alveolitis,” *Arthritis and Rheumatism*, vol. 43, no. 7, pp. 1633–1640, 2000.

[27] P. Borilova Linhartova, J. Kastovsky, S. Lucanova et al., “*Interleukin-17A* gene variability in patients with type 1 diabetes mellitus and chronic periodontitis: its correlation with IL-17 levels and the occurrence of periodontopathic bacteria,” *Mediators of Inflammation*, vol. 2016, Article ID 2979846, 9 pages, 2016.

[28] L. I. Holla, B. Hrdlickova, P. Linhartova, and A. Fassmann, “Interferon-γ +874A/T polymorphism in relation to generalized chronic periodontitis and the presence of periodontopathic bacteria,” *Archives of Oral Biology*, vol. 56, no. 2, pp. 153–158, 2011.

[29] W. J. Loesche and N. S. Grossman, “Periodontal disease as a specific, albeit chronic, infection: diagnosis and treatment,” *Clinical Microbiology Reviews*, vol. 14, no. 4, pp. 727–752, 2001.

[30] Ministry of Health CZ, “Public health report for CZ 2014,” 2018, November 2018, http://www.mzcr.cz/verejne/dokumenty/zprava-o-zdravi-obyvatel-ceske-republiky2014-_9420_3016_5.html.

[31] H. Kato, N. Tsuchiya, and K. Tokunaga, “Single nucleotide polymorphisms in the coding regions of human CXC-chemokine receptors CXCR1, CXCR2 and CXCR3,” *Genes and Immunity*, vol. 1, no. 5, pp. 330–337, 2000.

[32] M. C. Matheson, J. A. Ellis, J. Raven, E. H. Walters, and M. J. Abramson, “Association of IL8, CXCR2 and TNF-α polymorphisms and airway disease,” *Journal of Human Genetics*, vol. 51, no. 3, pp. 196–203, 2006.

[33] B. M. Ryan, A. I. Robles, A. C. McClary et al., “Identification of a functional SNP in the $3'$UTR of CXCR2 that is associated with reduced risk of lung cancer,” *Cancer Research*, vol. 75, no. 3, pp. 566–575, 2015.

[34] M. M. Barsante, T. M. Cunha, M. Allegretti et al., “Blockade of the chemokine receptor CXCR2 ameliorates adjuvant-induced arthritis in rats,” *British Journal of Pharmacology*, vol. 153, no. 5, pp. 992–1002, 2008.

[35] S. Almasi, M. R. Aliparasti, M. Farhoudi et al., “Quantitative evaluation of CXCL8 and its receptors (CXCR1 and CXCR2) gene expression in Iranian patients with multiple sclerosis,” *Immunological Investigations*, vol. 42, no. 8, pp. 737–748, 2013.

[36] J. Javor, M. Bucova, O. Cervenova et al., “Genetic variations of interleukin-8, CXCR1 and CXCR2 genes and risk of acute pyelonephritis in children,” *International Journal of Immunogenetics*, vol. 39, no. 4, pp. 338–345, 2012.

[37] A. C. Viana, Y. J. Kim, J. A. Cirelli et al., “A novel PCR-RFLP assay for the detection of the single nucleotide polymorphism at position +1440 in the human CXCR2 gene,” *Biochemical Genetics*, vol. 45, no. 9-10, pp. 737–741, 2007.

[38] “NCBI, dbSNP short genetic variations,” 2018, November 2018, https://www.ncbi.nlm.nih.gov/snp/rs1126580#frequency_tab.

[39] L. Nibali, A. Di Iorio, O. Onabolu, and G.-H. Lin, “Periodontal infectogenomics: systematic review of associations between host genetic variants and subgingival microbial detection,” *Journal of Clinical Periodontology*, vol. 43, no. 11, pp. 889–900, 2016.

[40] F. Cavalla, C. Biguetti, J. Lima Melchiades et al., “Genetic association with subgingival bacterial colonization in chronic periodontitis,” *Genes*, vol. 9, no. 6, p. 271, 2018.

[41] T. Cirelli, L. S. Finoti, S. C. T. Corbi et al., “Absolute quantification of *Aggregatibacter actinomycetemcomitans* in patients carrying haplotypes associated with susceptibility to chronic periodontitis: multifaceted evaluation with periodontitis covariants,” *Pathogens and Disease*, vol. 75, no. 7, 2017.

[42] J. Bartova, P. Borilova Linhartova, S. Podzimek et al., “The effect of IL-4 gene polymorphisms on cytokine production in patients with chronic periodontitis and in healthy controls,” *Mediators of Inflammation*, vol. 2014, Article ID 185757, 11 pages, 2014.

[43] E. J. Gaetti-Jardim Jr, T. C. Wahasugui, P. H. Tomazinho, M. M. Marques, V. Nakano, and M. J. Avila-Campos, “Distribution of biotypes and leukotoxic activity of *Aggregatibacter actinomycetemcomitans* isolated from Brazilian patients with chronic periodontitis,” *Brazilian Journal of Microbiology*, vol. 39, no. 4, pp. 658–663, 2008.

4.4 Comment to the article ”Interleukin-17A gene variability in patients with type 1 diabetes mellitus and chronic periodontitis: its correlation with IL-17 levels and the occurrence of periodontopathic bacteria”

Borilova Linhartova P, Kastovsky J, Lucanova S, Bartova J, Poskerova H, Vokurka J, Fassmann A, Kankova K, Izakovicova Holla L. Interleukin-17A gene variability in patients with type 1 diabetes mellitus and chronic periodontitis: its correlation with IL-17 levels and the occurrence of periodontopathic bacteria. Mediators Inflamm. 2016;2016:2979846. DOI: 10.1155/2016/2979846. (IF 3.232; CELL BIOLOGY Q2, IMMUNOLOGY Q2)

Author contribution: P.B.L. designed the study, drafted the paper, and particularly carried out genetic analysis.

IL-17 contributes to the pathogenesis of T1DM and CP. Neverthless, no previous study has investigated *IL-17* gene variability in T1DM patients so far and only a few studies have reported a relationship between polymorphisms in the *IL-17* gene and periodontitis with contradictory results (Corrêa et al., 2012; Kadkhodazadeh et al., 2013a,b; Saraiva et al., 2013; Zacarias et al., 2015).

The aim of this study was to investigate the possible association of *IL-17A* and *IL-17F* rs763780 gene variants with T1DM and/or CP. In addition, *IL-17A* SNP was examined in relation to the occurrence of selected periodontal bacteria in subgingival pockets and production of IL-17 by mononuclear cells in a subgroup of CP patients and a non-periodontitis HC.

In total 523 subjects were included in this case-control study. 125 T1DM patients, 244 CP patients and 154 non-periodontitis HC were genotyped using the TaqMan PCR method for *IL-17A* rs2275913 and *IL-17F* rs763780 polymorphisms. Subgingival bacterial colonization was analyzed with a DNA microarray detection kit in an external laboratory (Stomagene, České Budějovice, Czech Republic). The levels of IL-17 were measured using Luminex methods in subgroups of T1DM+CP patients, CP patients, and the non-periodontitis HC. Isolation, cultivation, and stimulation of PBMCs by selected periodontal bacteria (*A. actinomycetemcomitans, P. gingivalis*, *P. intermedia*, and *T. forsythia*), mitogens or HSP70, were described in our previous study (Bartova et al., 2014).

Although no differences in the allele/genotype frequencies between patients with CP and T1DM+CP were found, the *IL-17A* rs2275913 A allele increased the risk of T1DM ($P<0.05$, OR=1.36, 95%CI=0.96-1.92). *IL-17F* rs763780 TT genotype occurred in 93.7%

Carriers of *IL-17A* rs2275913 AA genotype had an increased risk of *T. forsythia* occurrence (in CP patients, P<0.05). In unstimulated PBMCs and also in cells stimulated by *P. gingivalis* of CP cariers with AA+AG genotypes a higher production of IL-17 was recorded than in GG homozygotes (*P*<0.05 in both). Production of IL-17 was significantly associated with *IL-17A* rs2275913 polymorphism in the unstimulated mononuclear cells from the pool of CP patients and the HC (*P*<0.05).

The recent meta-analysis by da Silva et al. (2017) showed a non-significant association between *IL-17A* rs2275913 or *IL-17F* rs763780 polymorphisms and CP in the allelic evaluation. Our study was included in the analysis and no bias of publication was observed by Egger's and Begg's tests in any allelic evaluation (da Silva et al., 2017).

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 2979846, 9 pages
http://dx.doi.org/10.1155/2016/2979846

Research Article

Interleukin-17A Gene Variability in Patients with Type 1 Diabetes Mellitus and Chronic Periodontitis: Its Correlation with IL-17 Levels and the Occurrence of Periodontopathic Bacteria

Petra Borilova Linhartova,[1,2] Jakub Kastovsky,[1,2] Svetlana Lucanova,[1] Jirina Bartova,[3] Hana Poskerova,[1] Jan Vokurka,[1] Antonin Fassmann,[1] Katerina Kankova,[2] and Lydie Izakovicova Holla[1,2]

[1] *Clinic of Stomatology, Institutions Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, 656 91 Brno, Czech Republic*
[2] *Department of Pathophysiology, Faculty of Medicine, Masaryk University, 625 00 Brno, Czech Republic*
[3] *Institute of Clinical and Experimental Dental Medicine, General University Hospital and First Faculty of Medicine, Charles University, 128 08 Prague, Czech Republic*

Correspondence should be addressed to Lydie Izakovicova Holla; holla@med.muni.cz

Received 25 September 2015; Revised 30 December 2015; Accepted 3 January 2016

Academic Editor: Kong Chen

Copyright © 2016 Petra Borilova Linhartova et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Interleukin-17 contributes to the pathogenesis of type 1 diabetes mellitus (T1DM) and chronic periodontitis (CP). We analyzed *IL-17A* −197A/G and *IL-17F* +7488C/T polymorphisms in T1DM and CP and determined their associations with IL-17 production and occurrence of periopathogens. Totally 154 controls, 125 T1DM, and 244 CP patients were genotyped using 5′ nuclease TaqMan® assays. Bacterial colonization was investigated by a DNA-microarray kit. Production of IL-17 after *in vitro* stimulation of mononuclear cells by mitogens and bacteria was examined by the Luminex system. Although no differences in the allele/genotype frequencies between patients with CP and T1DM + CP were found, the *IL-17A* −197 A allele increased the risk of T1DM ($P < 0.05$). Levels of HbA1c were significantly elevated in carriers of the A allele in T1DM patients ($P < 0.05$). Production of IL-17 by mononuclear cells of CP patients (unstimulated/stimulated by *Porphyromonas gingivalis*) was associated with *IL-17A* A allele ($P < 0.05$). *IL-17A* polymorphism increased the number of *Tannerella forsythia* and *Treponema denticola* in patients with CP and T1DM + CP, respectively ($P < 0.05$). *IL-17A* gene variability may influence control of T1DM and the "red complex" bacteria occurrence in patients with CP and T1DM + CP. Our findings demonstrated the functional relevance of the *IL-17A* polymorphism with higher IL-17 secretion in individuals with A allele.

1. Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease caused by T cell-mediated destruction of pancreatic β cells resulting in the absence of insulin and uncontrolled hyperglycemia. A complex interplay between genetic and environmental factors participates in developing of T1DM and manifestation of its systemic and oral complications. Periodontitis has been identified as the sixth complication of diabetes; the other five complications are retinopathy (DR), nephropathy (DN), neuropathy (DPN), macrovascular disease, and poor wound healing [1]. Periodontitis is a chronic infection characterized by progressive inflammatory response to bacteria in dental plaque, which finally results in periodontal tissue destruction and tooth loss. The relationship between diabetes mellitus and periodontitis has been reported previously [2]. Chronic hyperglycemia induces a proinflammatory state in the gingival microcirculation characterized by an increased vascular permeability, and leukocyte and endothelial cell activation, which may contribute to periodontal tissue damage

in diabetes mellitus [3]. Chronic periodontitis (CP) is more frequent in T1DM patients and can worsen its metabolic control [4, 5]. Nevertheless, molecular mechanisms responsible for periodontal disease and its progression in T1DM patients remain unknown.

Although individuals at risk for T1DM are recognized by screening for HLA-associated risk genotypes and β cell autoantibodies, recently the pathogenic role of IL-17-secreting T helper 17 (Th17) cells has been implicated in the development of T1DM [6–8]. The IL-17 cytokine family consists of six cytokine members designed from IL-17A (originally cloned and named CTLA-8), IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F, according to the order of their discoveries. IL-17A and IL-17F, the most studied members in the IL-17 family, are located close to each other on human chromosome 6, sharing the highest amino acid sequence identity (50%) and similar functions. They are primarily involved in autoimmune responses, tumor development, and host defense against bacterial and fungal infections by activating epithelial innate immune responses and production of antimicrobial peptides, cytokines (e.g., IL-6 or TNF-α), chemokines (e.g., IL-8), and cartilage degrading metalloproteinases as well as cytokines promoting osteoclastogenesis that results in bone destruction [9–11].

Several lines of evidence suggest that IL-17 plays a role in human diabetes. Bradshaw et al. [12] observed that monocytes isolated from T1DM patients induced more IL-17 producing T cells compared with healthy controls. They also observed significantly increased IL-17 producing T cells in peripheral blood of patients with long standing T1DM [12]. Arif et al. [13] demonstrated that activation of IL-17 pathway accelerated pancreatic β cell apoptosis and led to autoimmune diabetes. They observed significantly elevated IL-17A expression in pancreas from newly diagnosed T1DM patients. In addition, peripheral blood lymphocytes from T1DM patients had elevated IL-17A and IL-17F expression [14].

The emerging role of IL-17 in periodontal disease was also discussed in a recent study, based on evidence from human and animal models [15]. Upregulated *IL-17A* gene expression has been observed in patients with CP, suggesting that the net effect of IL-17 signaling promotes the disease development [16–22]. Additionally, IL-17RA deficient mice were found more susceptible to *Porphyromonas gingivalis* (*P.g.*), causal Gram-negative bacteria of the "red complex" [23, 24]. Based on the fact that increased IL-17 levels occur in the gingival tissue of patients with periodontal disease [25–27], Park et al. [28] hypothesized that *P.g.* lipopolysaccharide (LPS) might mediate IL-17 release from human periodontal ligament cells.

Despite the important role of IL-17 cytokine in T1DM and CP pathogenesis, no study has investigated *IL-17* gene variability in T1DM patients so far and only a few studies have reported a relationship between polymorphisms in the *IL-17* gene and periodontitis with contradictory results [29–33]. Therefore, in the present study, we aimed to investigate the association of *IL-17A* −197A/G (rs2275913) and *IL-17F* +7488C/T (His161Arg, rs763780) gene polymorphisms with T1DM and/or CP. In addition, *IL-17A* single nucleotide polymorphism (SNP) was examined in relation to the occurrence of selected periodontal bacteria in subgingival pockets and production of IL-17 by mononuclear cells in a subgroup of CP patients and healthy controls.

2. Material and Methods

The study was performed with the approval of the Committees for Ethics of the Medical Faculty, Masaryk University Brno and St. Anne's Faculty Hospital. Written informed consent was obtained from all participants in line with the Helsinki declaration before inclusion in the study.

2.1. Study Population and Clinical Examinations. In this case-control study, 523 unrelated adult subjects from the Czech Republic were included. One hundred and twenty-five patients with T1DM were followed in the outpatient unit of the Diabetology Clinics in South Moravia Region, Czech Republic. The diagnosis of T1DM was originally based on the presence of clinical symptoms (such as polyuria, polydipsia, and weight loss) and biochemical parameters (glycemia, ketoacidosis, and autoantibody status). All patients were receiving intensified insulin therapy or insulin pump and other medicaments according to the presence of diabetic complications, such as DN, DR, DPN, and other comorbidities as described on a part of our cohort previously [34]. Duration of diabetes was defined as the period from diabetes onset until the enrolment in this study. Levels of glycemia, glycated hemoglobin (HbA1c), total cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), body mass index (BMI), and further parameters were recorded. The periodontal status was evaluated in a subgroup of 38 diabetic patients, 154 healthy controls, and 244 CP subjects recruited from a patient pool of the Clinic of Stomatology, St. Anne's Faculty Hospital Brno, from 2005 to 2015. The controls were selected from subjects referred to the Clinic of Stomatology for reasons other than periodontal disease (such as dental caries, orthodontic consultations, and preventive dental checkups) during the same period as patients and matched for age and gender. Exclusion criteria were history of systemic diseases such as cardiovascular disorders (e.g., coronary artery diseases), diabetes mellitus, malignant diseases, immunodeficiency, and current pregnancy or lactation.

The diagnosis of periodontitis/nonperiodontitis was based on the detailed clinical examination, medical and dental history, tooth mobility, and radiographic assessment as described in our previous study [35].

2.2. Genetic Analysis. Isolation and storage of DNA and genotyping of samples were conducted in the laboratory of the Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.

Genotyping of two SNPs in *IL-17*, *IL-17A* −197A/G (rs2275913), and *IL-17F* +7488C/T (His161Arg, rs763780) was based on polymerase chain reaction using $5'$ nuclease TaqMan assays (C_15879983_10, C_2234166_10, resp.). Reaction mixture and conditions were designed according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA) and fluorescence was measured using the ABI PRISM 7000 Sequence Detection System. SDS version 1.2.3

Table 1: Demographic data of the healthy controls and the studied subjects with CP, T1DM (and the T1DM + CP subgroup).

Characteristics	Controls $N = 154$	CP $N = 244$	T1DM $N = 125$	T1DM + CP $N = 38$
Age (mean years ± SD)	48.5 ± 10.7	52.5 ± 9.8	46.4 ± 13.8	49.9 ± 10.6
Sex (males/females)	75/79	112/132	67/58	16/22
Smoking (no/yes, %)	71.1/28.9	73.9/26.1	78.9/21.1[#]	78.9/21.1
BMI (mean ± SD)	23.2 ± 4.6	26.4 ± 3.7	25.0 ± 4.9	25.3 ± 3.1
Duration of DM (mean years ± SD)	—	—	22.8 ± 10.3	24.0 ± 11.3
HbA1c (mmol/mol, mean ± SD)	—	—	76.5 ± 17.3	69.9 ± 11.4
DN (no/yes, %)	—	—	52.9/47.1	71.9/28.1
DR (no/yes, %)	—	—	30.4/69.6	42.4/57.6
DPN (no/yes, %)	—	—	39.3/60.7	47.1/52.9

BMI = body mass index, CP = chronic periodontitis, DN = diabetic nephropathy, DPN = diabetic peripheral neuropathy, DR = diabetic retinopathy, HbA1c = glycated hemoglobin, N = number of subjects, SD = standard deviation, and T1DM = type 1 diabetes mellitus.
[#]Smoking status is known only in T1DM patients with CP.

software was used to analyze real-time and endpoint fluorescence data.

2.3. DNA-Microarray Analyses of Oral Pathogens. DNA-microarray analyses of oral pathogens based on a periodontal pathogen detection kit (Protean Ltd., Ceske Budejovice, Czech Republic) were previously used and described [35]. Bacterial colonization [*Aggregatibacter actinomycetemcomitans* (*A.a.*), *Tannerella forsythia* (*T.f.*), *P.g.*, *Prevotella intermedia* (*P.i.*), *Treponema denticola* (*T.d.*), *Peptostreptococcus micros* (*P.i.*), and *Fusobacterium nucleatum* (*F.n.*)] in subgingival sulci/pockets was investigated in a subgroup of controls ($N = 51$), CP patients ($N = 182$), and T1DM patients with CP ($N = 38$) before subgingival scaling. This test determined the individual pathogens semiquantitatively as follows: (−) undetected, which corresponds to the number of bacteria less than 10^3, (+) slightly positive corresponding to the number of bacteria 10^3 to 10^4, (++) positive corresponding to the number of bacteria 10^4 to 10^5, and (+++) strongly positive, with the number of bacteria higher than 10^5.

2.4. Cultivation of Bacteria and Immunological Examination. Cultivation of periodontopathic bacteria and immunological examination were performed in the laboratory of the Institute of Clinical and Experimental Dental Medicine, General University Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic.

IL-17 levels were measured in a subgroup of 15 healthy controls and 30 patients with CP. IL-17 levels were determined in mononuclear cells isolated from 20 mL of heparinized blood using the Luminex multiplex method (Luminex 100TM analyzer, R&D systems, USA). The isolation, cultivation, and stimulation of cells by selected bacteria (*A.a.*, *T.f.*, *P.g.*, and *P.i.*) and mitogens or Heat Shock Protein (HSP) 70 were described in detail in our previous article [36].

2.5. Statistical Analysis. Statistical analysis was performed using the statistical package Statistica v. 10 (StatSoft Inc., USA). Standard descriptive statistics were applied in the analysis: absolute and relative frequencies for categorical variables and mean with standard deviation (SD) or median with quartiles for quantitative variables. To compare independent groups, one-way analysis of variance (ANOVA) and Kruskal-Wallis (ANOVA) were performed to compare continuous variables. The allele frequencies were calculated from the observed numbers of genotypes. The differences in the allele frequencies were tested by the Fisher-exact test; Hardy-Weinberg equilibrium (HWE) and genotype frequencies were calculated by the chi-square test (χ^2). The association was described by odds ratios (OR) with 95% confidence intervals (95% CI). Only the values of P less than 0.05 were considered as statistically significant.

3. Results

3.1. Case-Control Study. The demographic data of the study population are shown in Table 1. The mean ages and BMI between the healthy controls and patient groups did not differ significantly ($P > 0.05$). There were also no significant differences between the subjects with T1DM or CP and the controls relating to males/females ratio or smoking status. All diabetic patients ($N = 38$) who were examined at the Periodontology Department were affected by periodontitis. Duration of DM and diabetic complications (DN, DR, and DPN) in the whole diabetic cohort versus subgroup of T1DM patients with CP were not statistically different.

3.2. SNPs Analysis. Both studied polymorphisms were in the HWE in the control group ($P > 0.05$). Considering the fact that, in the Czech population, SNP *IL-17F* +7488C/T (His161Arg, rs763780) TT genotype occurred in 93.7%, we analyzed this polymorphism only in the subgroup of subjects ($N = 190$, data not shown).

The allele and genotype frequencies of the *IL-17A* −197A/G (rs2275913) variant are shown in Table 2. While the genotype frequencies were not different between the controls and CP or T1DM patients, the A allele was marginally associated with an increased risk to T1DM ($P < 0.05$,

Table 2: *IL-17A* −197A/G (rs2275913) genotype and allele frequencies in healthy controls, patients with CP, T1DM (and the T1DM + CP subgroup).

IL-17A −197	Controls	CP			T1DM			T1DM + CP		
Genotype and allele	$N = 154$ (%)	$N = 244$ (%)	P	OR (95% CI)	$N = 125$ (%)	P	OR (95% CI)	$N = 38$ (%)	P	OR (95% CI)
AA	18 (11.7)	32 (13.1)	0.56	1.00 (0.52–1.93)	23 (18.4)	0.05	1.93 (0.93–4.00)	6 (15.8)	0.46	1.20 (0.42–3.48)
AG	71 (46.1)	97 (39.8)	0.14	0.77 (0.50–1.19)	59 (47.2)	0.23	1.26 (0.75–2.11)	14 (36.8)	0.25	0.71 (0.33–1.55)
GG	65 (42.2)	115 (47.1)		1.00	43 (34.4)		1.00	18 (47.4)		1.00
AA + AG versus GG	89 versus 65 (57.8/42.2)	129 versus 115 (52.9/47.1)	0.20	0.82 (0.55–1.23)	82 versus 43 (65.6/34.4)	0.11	1.39 (0.85–2.27)	20 versus 18 (52.6/47.4)	0.35	0.81 (0.40–1.65)
A	107 (34.7)	161 (33.0)		0.92 (0.68–1.25)	105 (42.0)		1.36 (0.96–1.92)	26 (34.2)		0.98 (0.58–1.66)
G	201 (65.3)	327 (67.0)	0.33	1.00	145 (58.0)	0.05*	1.00	50 (65.8)	0.37	1.00

CI = confidential interval, CP = chronic periodontitis, N = number of subjects, OR = odds ratio, and T1DM = diabetes mellitus type 1; * P value < 0.05 (in comparison with healthy controls).

Table 3: Levels of HbA1c in correlation with *IL-17A* −197A/G (rs2275913) genotypes in T1DM#.

IL-17A −197	T1DM		
Genotype	N	HbA1c (mmol/mol) Mean ± SD	P
AA + AG	69	76.6 ± 16.5	
GG	35	69.8 ± 13.9	0.03*

HbA1c = glycated hemoglobin, N = number of subjects, SD = standard deviation, and T1DM = type 1 diabetes mellitus; * P value < 0.05 (parametric test, ANOVA).

#Levels of HbA1c were available in 104 patients with T1DM.

OR = 1.36, 95% CI = 0.96–1.92). Moreover, mean levels of HbA1c were significantly elevated in carriers of the A allele (AA and AG genotypes in comparison to GG homozygotes) in a group of T1DM patients (76.6 mmol/mol versus 69.8 mmol/mol, $P < 0.05$, Table 3).

Although no significant differences in the *IL-17A* allele or genotype frequencies between patients with CP and healthy controls were found in the whole set (Table 2), stratification of subjects according to smoking status revealed the following differences: in healthy subjects, the A allele frequency was higher in smokers ($P < 0.05$, OR = 1.62, 95% CI = 0.97–2.70), whereas, in CP patients, this allele was found more frequently in nonsmokers ($P < 0.05$, OR = 0.64, 95% CI = 0.41–1.02). Although a similar distribution of alleles or genotypes between the groups of control nonsmokers versus CP nonsmokers was found, frequencies of the A allele ($P < 0.01$, OR = 2.14, 95% CI = 1.19–3.83), AA genotype ($P < 0.05$, OR = 3.17, 65% CI = 0.89–11.28), and AA + AG versus GG genotypes ($P < 0.05$, OR = 1.82, 95% CI = 0.81–4.09) were more frequent in the control smokers in comparison with the smokers with CP (Table 4). However, as the associations detected between the given subgroups were only of borderline significance and numbers of the individuals in the subgroups were small, the results obtained should be interpreted carefully. A subanalysis performed separately in the groups of females ($N = 269$) and males ($N = 254$) showed no significant difference in the *IL-17A* −197A/G (rs2275913) allele or genotype frequencies (data not shown). Due to the small number of T1DM patients with confirmed CP, this group was not analyzed according to gender or smoking status.

3.3. Microbial Analysis. Possible links between the *IL-17A* −197A/G (rs2275913) variant and the occurrence of seven selected periodontal bacteria in subgingival pockets were analyzed. The *IL-17A* −197 A allele carriers had an increased risk of *T.f.* occurrence in 182 CP patients (34.9% versus 19.6%, $P < 0.05$, OR = 2.20, 95% CI = 1.03–4.73) and a similar but nonsignificant trend was observed for the presence of *P.i.* (36.0% versus 28.7%, $P = 0.09$, OR = 1.40, 95% CI = 0.89–2.20). On the other hand, in patients with T1DM and CP carrying A allele of the *IL-17A* −197 polymorphism, *T.d.* in subgingival biofilm occurred less frequently than in subjects without this allele (26.9% versus 50.0%, $P < 0.05$, OR = 0.37, 95% CI = 0.13–1.01, Table 5). In subgroups of nonsmokers, the *IL-17A* −197 A allele carriers had an increased risk for the occurrence of *P.i.* in patients with CP only (39.6% versus 28.7%, $P < 0.05$, OR = 1.63, 95% CI = 0.96–2.76, Table 5). The *IL-17A* −197 SNP was not associated with the occurrence of any other bacteria (including *P.g.*), from those seven selected ones (data not shown).

3.4. Immunological Analysis. We analyzed IL-17 levels in relation to the *IL-17A* −197A/G (rs2275913) polymorphism in selected patients with CP and healthy controls. The IL-17 levels were measured after a 3-day *in vitro* cultivation of mononuclear cells, without or with stimulation by dental plaque bacteria, mitogens, or HSP70 in the CP patients ($N = 30$). Carriers of genotype with the A allele of *IL-17A* −197A/G (rs2275913) SNP had a higher production of IL-17 by unstimulated monocytes (0.98 pg/mL versus 0.27 pg/mL, $P < 0.05$) and also after stimulation with *P.g.* (1.51 pg/mL versus 0.10 pg/mL, $P < 0.05$) than GG homozygotes (Table 6).

TABLE 4: *IL-17A* −197A/G (rs2275913) genotype and allele frequencies in healthy controls and patients with CP stratified by smoking status.

IL-17A −197	Control nonsmokers	Control smokers		CP nonsmokers	CP smokers			
Genotype and allele	$N = 108$ (%)	$N = 44$ (%)	P	$N = 173$ (%)	$N = 61$ (%)	P	P^a	P^b
AA	10 (9.2)	8 (18.2)		28 (16.2)	4 (6.6)			
AG	49 (45.4)	22 (50.0)		67 (38.7)	24 (39.3)			
GG	49 (45.4)	14 (31.8)	0.16	78 (45.1)	33 (54.1)	0.15	0.22	0.04*
AA + AG versus GG	59 versus 49 (54.6/45.4)	30 versus 14 (68.2/31.8)	0.09	95 versus 78 (54.9/45.1)	28 versus 33 (45.9/54.1)	0.14	0.10	0.02*
A	69 (31.9)	38 (43.2)		123 (35.5)	32 (26.2)			
G	147 (68.1)	50 (56.8)	0.04*	223 (64.5)	90 (73.8)	0.04*	0.22	0.01*

CP = chronic periodontitis, N = number of subjects, and * P value < 0.05.
P^a comparison of groups of nonsmokers.
P^b comparison of groups of smokers.

TABLE 5: The presence of bacteria in correlation with *IL-17A* −197A/G (rs2275913) polymorphism in patients with CP and T1DM + CP#.

IL-17A −197	CP $N = 182$ (%)			CP $N = 182$ (%)			T1DM + CP $N = 38$ (%)		
Genotype and allele	*T.f.* neg	*T.f.* pos	P	*P.i.* neg	*P.i.* pos	P	*T.d.* neg	*T.d.* pos	P
AA	0 (0.0)	24 (15.1)	0.02*	6 (8.0)	18 (16.8)	0.07	3 (25.0)	3 (11.5)	0.14
AG	9 (39.1)	63 (39.6)	0.33	31 (41.3)	41 (38.3)	0.51	6 (50.0)	8 (30.8)	0.11
GG	14 (60.9)	72 (45.3)		38 (50.7)	48 (44.9)		3 (25.0)	15 (57.7)	
AA + AG versus GG	9 versus 14 (39.1 versus 60.9)	87 versus 72 (54.7 versus 45.3)	0.12	37 versus 38 (49.3 versus 50.7)	59 versus 48 (55.1 versus 44.9)	0.27	9 versus 3 (75.0 versus 25.0)	11 versus 15 (42.3 versus 57.7)	0.06
A	9 (19.6)	111 (34.9)		43 (28.7)	77 (36.0)		12 (50.0)	14 (26.9)	
G	37 (80.4)	207 (65.1)	0.03*	107 (71.3)	137 (64.0)	0.09	12 (50.0)	38 (73.1)	0.04*
IL-17A −197	CP nonsmokers $N = 131$ (%)			CP nonsmokers $N = 131$ (%)			T1DM + CP nonsmokers $N = 30$ (%)		
Genotype and allele	*T.f.* neg	*T.f.* pos	P	*P.i.* neg	*P.i.* pos	P	*T.d.* neg	*T.d.* pos	P
AA	0 (0.0)	20 (17.5)	0.06	4 (7.4)	16 (20.8)	0.04*	2 (25.0)	3 (13.6)	0.27
AG	8 (47.1)	44 (38.6)	0.59	23 (42.6)	29 (37.7)	0.51	4 (50.0)	7 (31.8)	0.21
GG	9 (52.9)	50 (43.9)		27 (50.0)	32 (41.6)		2 (25.0)	12 (54.5)	
AA + AG versus GG	8 versus 9 (47.1 versus 52.9)	64 versus 50 (56.1 versus 43.9)	0.33	27 versus 27 (50.0 versus 50.0)	45 versus 32 (58.5 versus 41.6)	0.22	6 versus 2 (75.0 versus 25.0)	10 versus 12 (45.4 versus 54.5)	0.15
A	8 (23.5)	84 (36.8)		31 (28.7)	61 (39.6)		8 (50.0)	13 (29.5)	
G	26 (76.5)	144 (63.2)	0.09	77 (71.3)	93 (60.4)	0.05*	8 (50.0)	31 (70.5)	0.12

CP = chronic periodontitis, N = number of subjects, neg = negative, pos = positive, T1DM = type 1 diabetes mellitus, *P.i.* = *Prevotella intermedia*, *T.d.* = *Treponema denticola*, *T.f.* = *Tannerella forsythia*, and * P value < 0.05.
#Of the seven periodontal pathogens analyzed, only those with significant differences are shown.

In the healthy controls ($N = 15$), unstimulated IL-17 levels were 0.10 (0.08–0.26) pg/mL (median; 25–75 quartiles) without any significant relationship with *IL-17A* polymorphism. After pooling both groups ($N = 45$), production of IL-17 was significantly associated with *IL-17A* polymorphism in the unstimulated mononuclear cells ($P < 0.05$), but not after stimulation by *P.g.* ($P = 0.06$, data not shown). *IL-17A* −197A/G SNP had no significant effect on IL-17 production after stimulation with other periodontal bacteria, HSP70, and/or mitogens (data not shown).

4. Discussion

Inflammation is a physiological immune response triggered during infection and injury in an attempt to prevent infection and promote regeneration. However, persistent and

Table 6: Levels of IL-17 in correlation with *IL-17A* −197A/G (rs2275913) genotypes in patients with CP[#].

IL-17A −197		IL-17 unstimulated (pg/mL); median (25–75 quartiles)	
Genotype	*N*		*P*
AA + AG	19	0.98 (0.25–8.53)	
GG	11	0.27 (0.00–0.54)	0.04*
IL-17A −197		IL-17 stimulated by *P.g.* (pg/mL); median (25–75 quartiles)	
Genotype	*N*		*P*
AA + AG	19	1.51 (0.50–4.56)	
GG	11	0.10 (0.00–1.51)	0.02*

CP = chronic periodontitis, *N* = number of subjects, * P value < 0.05 (Kruskal-Wallis test, ANOVA), and *P.g.* = *Porphyromonas gingivalis.*
Levels of IL-17 were available in 30 patients with CP.
[#]Only significant differences were shown.

unwarranted inflammation can result in host tissue damage [8]. Regulation of inflammation is a complex process, tightly controlled by signaling messengers of the immune system, such as cytokines. IL-17A and IL-17F, produced mainly by Th17 cells, have been found to be involved in the pathogenesis of autoimmune diseases including diabetes and chronic inflammation, such as periodontitis [12, 15, 37–41].

We evaluated *IL-17A* −197A/G (rs2275913) and *IL-17F* +7488C/T (His161Arg, rs763780) SNPs in a group of adults with and without T1DM and/or CP from Czech population. As the present study identified a low variability of *IL-17F* at position +7488C/T (similarly as other studies in other populations: http://www.snpedia.com/index.php/Rs763780), the TT genotype occurred in 93.7%, and no CC homozygote was detected, we investigated this polymorphism only in a subgroup of 190 subjects. Further, no associations of this polymorphism with aggressive periodontitis (AgP) or CP [31–33] have been previously found. In contrast, the *IL-17A* −197A/G SNP was analyzed in the whole set of our 523 subjects. The minor allele frequency (MAF) of this SNP was 0.36, which is in line with the allele frequency in other European population [42]. To this date, no study investigating an association of the *IL-17A* −197A/G or *IL-17F* +7488C/T variants with T1DM or periodontitis in European white population has been published. Although no significant differences were found in the genotype frequencies between the healthy subjects and T1DM or CP patients, the A allele was marginally associated with an increased risk of T1DM ($P < 0.05$). This allele displayed a higher affinity for the nuclear factor of activated T cells (NFAT), a critical transcription factor involved in the IL-17 regulation [43, 44]. Espinoza et al. [43] reported that healthy individuals possessing the A allele of *IL-17A* −197A/G (rs2275913) produced significantly more IL-17 after *in vitro* T cells stimulation than those without this allele. Shao et al. [45] described that uncontrolled expansion of Th17 cells was involved in T1DM pathology and might exert essential effects on its development.

In the studied diabetic population, significantly higher levels of HbA1c were present in T1DM carriers of the genotype with the A allele (AA + AG genotypes) versus the GG homozygotes (76.6 mmol/mol ± 16.5 mmol/mol versus 69.8 mmol/mol ± 13.9 mmol/mol). To our knowledge, no previous study has focused on this issue; however, polymorphisms in other genes, for example, *IL-6* [46] and *IL-18* separately [47] or in combination with the *IL-12B* gene [48], have been associated with higher concentration of HbA1c in T1DM populations.

In addition, we found no significant differences in the *IL-17A* −197A/G (rs2275913) allele or genotype frequencies between the healthy subjects and patients with CP, not even after stratification by sex (data not shown). No studies on this topic from European but only from Iranian and Brazilian populations have been reported. In the Iranian population, the CC genotype of another *IL-17A* variant (rs10484879) was associated with CP and peri-implantitis [29]. Three Brazilian studies examined variability in the *IL-17A* −197A/G (rs2275913) gene in relation to periodontal disease with controversial results. In the study by Saraiva et al. [32], the A allele was associated with the absence of periodontitis, but Corrêa et al. [31] and Zacarias et al. [33] found the AA genotype and the A allele as a risk factor for CP. Even in the separate subgroup of Czech patients with CP and T1DM, no relationship between *IL-17A* polymorphism and periodontal status (PD = probing pocket depth, CAL = clinical attachment loss, etc.) was found. However, Gürsoy et al. [49] recently described the association between PD and IL-17 levels in saliva of type 2 diabetic patients, but independently of glycemic status. Interestingly, the allele and genotype distributions of the *IL-17A* variant in the subgroup of T1DM patients with CP were closer to the values in nondiabetic CP than T1DM patients. The different findings in Czech population may be due to differences in European versus Brazilian populations.

In contrast to findings in the Brazilian cohort where the AA genotype was identified as a risk factor for CP in non-smoker Caucasians [33], our results showed no differences in distribution of the *IL-17A* −197A/G alleles or genotypes between healthy and periodontitis nonsmokers. However, the G allele and the GG genotype were marginally significantly associated with an increased risk of periodontitis in smokers. Analysis of the allele frequencies in nonsmokers versus smokers showed borderline significant differences in both studied groups but in the opposite trend, in which 68.1% versus 56.8% for healthy controls and 64.5% versus 73.8% for patients with CP. Due to a relatively small number of subjects in the individual subgroups, number of comparisons

performed, and only marginally significant differences, our results should be interpreted carefully.

In the next step, according to the hypothesis about biological functions and regulation of IL-17 which plays a role in host defense [50], we assessed the *IL-17A* gene variability in relation to the presence of periodontopathic bacteria in subgingival pockets in patients with CP and T1DM + CP. In CP population, *IL-17A* –197 A allele carriers had an increased risk of *T.f.* occurrence and, in CP nonsmokers only, this allele increased risk of the occurrence of *P.i.*, but the same A allele was protective for the presence of *T.d.* in subgingival biofilm in T1DM patients with CP. In connection with periodontopathic bacteria, gene variability has been investigated in a few other interleukins to this date. The specific *IL-8* variants were associated with subgingival colonization with *A.a.* in AgP and *T.f.* in CP in the Czech population [35]. Finoti et al. [51] also found that *IL-8* haplotype influenced the presence of the "red complex" bacteria in gingival sulci. Several studies by Nibali et al. [52–56] focused on a correlation between the pathogenic bacterial colonization and variability in *IL-6* and *Fc γ receptor* genes. Additionally, SNPs in the *IL-1* gene cluster [57], *interferon γ*, and *IL-2* were associated with the presence of various periodontopathic bacteria, especially *A.a.* and the "red complex" bacteria. However, in our study, no other bacteria from those studied were associated with *IL-17A* –197 variant and there were no significant differences in the occurrence of these bacteria among groups.

Finally, in functional study, we associated IL-17 production *in vitro* by blood mononuclear cells with the *IL-17A* –197A/G (rs2275913) gene polymorphism in the subgroup of patients with CP. In Czech periodontitis patients, *IL-17A* –197 AA + AG carriers had higher IL-17 levels in unstimulated mononuclear cells than GG homozygotes. Our results are in accordance with the conclusion of Espinoza et al. [43], who connected the A allele with increased IL-17 levels *in vitro* after T cells stimulation. Even greater differences in IL-17 production were measured in CP carriers with the A allele in genotype after stimulation with *P.g.* It is in agreement with previous findings that *P.g.* LPS is a mediator of IL-17 release from human periodontal ligament cells [28]. Also Moutsopoulos et al. [58] proved that *P.g.* induced innate cell IL-17 production and promoted Th17 polarization. In a pooled group of CP and healthy subjects ($N = 45$), production of IL-17 was associated with the *IL-17A* polymorphism only in unstimulated mononuclear cells, but not after stimulation by *P.g.*, other periodontal bacteria, HSP70, and/or mitogens. In addition, recently Azman et al. [20] demonstrated that serum, saliva, and gingival crevicular fluid, IL-17A levels were higher in periodontitis patients and correlated positively with clinical parameters (PD, CAL, and BOP = bleeding on probing). Findings of the present study also demonstrated increased IL-17 levels in unstimulated monocytes in patients with periodontitis versus healthy controls (0.48 pg/mL versus 0.10 pg/mL; median). However, the comparison of absolute values of IL-17 levels in healthy subjects with results of other studies would require the use of the same method for the cytokine determination (including a kit from the same supplier), type and preparation of samples, and age of subjects [59, 60].

There are many possible limitations in this study. The present study is mainly limited by relatively low numbers of subjects and especially by the fact that from the group of 125 T1DM subjects, only 38 patients were evaluated for periodontal status. The negative findings of single marker analysis in this subgroup could be a result of a lack of statistical power to detect minor differences (small effect of genes/gene variants in multifactorial diseases is typical). In addition, most of the associations found were tightly below statistical significance ($P < 0.05$) without multitest corrections. Therefore, risk of relatively high false discovery rate means that our results should be interpreted with caution. Secondly, levels of IL-17 in plasma or in gingival tissue were not measured and the presence of periodontal pathogens was examined only in the 271 subjects. However, this study also has several strengths. This is the first study of the *IL-17* gene polymorphisms in T1DM population and in European patients with CP that was conducted in a relatively homogenous population of white Caucasians in Central Europe of the Czech origin. Secondly, the size of studied healthy subjects and CP patients is greater than in previous Iranian or Brazilian studies. Thirdly, we examined not only the polymorphisms alone but their relationship with clinical, bacterial, and biochemical parameters, which allowed a better biological assessment of the detected associations.

5. Conclusions

In conclusion, *IL-17A* gene variability may partially influence T1DM control and the "red complex" bacteria occurrence in patients with CP and diabetic patients with CP. Additionally, our findings confirmed the functional relevance of the *IL-17A* polymorphism with higher IL-17 secretion in the individuals with the A allele. However, the results of this study need to be proven in a larger independent cohort.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Petra Borilova Linhartova, Jirina Bartova, Katerina Kankova, and Lydie Izakovicova Holla designed the study and drafted the paper. Jirina Bartova, Hana Poskerova, Jan Vokurka, and Antonin Fassmann performed the clinical analysis and collected the blood samples. Jakub Kastovsky, Svetlana Lucanova, and Petra Borilova Linhartova carried out the molecular analysis, and Lydie Izakovicova Holla performed statistical analysis. All authors revised the final version of the paper.

Acknowledgments

The study was supported by Grants GACR GB14-37368G and IGA NT11405/6 and Project MUNI/A/1359/2014.

References

[1] H. Löe, "Periodontal disease. The sixth complication of diabetes mellitus," *Diabetes Care*, vol. 16, no. 1, pp. 329–334, 1993.

[2] B. L. Mealey, "Periodontal disease and diabetes. A two-way street," *Journal of the American Dental Association*, vol. 137, supplement, pp. 26S–31S, 2006.

[3] C. Sima, K. Rhourida, T. E. Van Dyke, and R. Gyurko, "Type 1 diabetes predisposes to enhanced gingival leukocyte margination and macromolecule extravasation *in vivo*," *Journal of Periodontal Research*, vol. 45, no. 6, pp. 748–756, 2010.

[4] F.-J. Silvestre, L. Miralles, F. Llambes, D. Bautista, E. Solá-Izquierdo, and A. Hernández-Mijares, "Type 1 diabetes mellitus and periodontal disease: relationship to different clinical variables," *Medicina Oral, Patologia Oral y Cirugia Bucal*, vol. 14, no. 4, pp. E175–E179, 2009.

[5] J. Sakalauskiene, R. Kubilius, A. Gleiznys, A. Vitkauskiene, E. Ivanauskiene, and V. Šaferis, "Relationship of clinical and microbiological variables in patients with type 1 diabetes mellitus and periodontitis," *Medical Science Monitor*, vol. 20, pp. 1871–1877, 2014.

[6] S. K. Bedoya, B. Lam, K. Lau, and J. Larkin III, "Th17 cells in immunity and autoimmunity," *Clinical and Developmental Immunology*, vol. 2013, Article ID 986789, 16 pages, 2013.

[7] A. K. Marwaha, S. Tan, and J. P. Dutz, "Targeting the IL-17/IFN-γ axis as a potential new clinical therapy for type 1 diabetes," *Clinical Immunology*, vol. 154, no. 1, pp. 84–89, 2014.

[8] P. Kumar and G. Subramaniyam, "Molecular underpinnings of Th17 immune-regulation and their implications in autoimmune diabetes," *Cytokine*, vol. 71, no. 2, pp. 366–376, 2015.

[9] Y. Iwakura, S. Nakae, S. Saijo, and H. Ishigame, "The roles of IL-17A in inflammatory immune responses and host defense against pathogens," *Immunological Reviews*, vol. 226, no. 1, pp. 57–79, 2008.

[10] Y. Iwakura, H. Ishigame, S. Saijo, and S. Nakae, "Functional specialization of interleukin-17 family members," *Immunity*, vol. 34, no. 2, pp. 149–162, 2011.

[11] D. Boroń, S. Agnieszka, K. Daniel, B. Anna, and K. Adam, "Polymorphism of interleukin-17 and its relation to mineral density of bones in perimenopausal women," *European Journal of Medical Research*, vol. 19, no. 1, article 69, 2014.

[12] E. M. Bradshaw, K. Raddassi, W. Elyaman et al., "Monocytes from patients with type 1 diabetes spontaneously secrete proinflammatory cytokines inducing Th17 cells," *The Journal of Immunology*, vol. 183, no. 7, pp. 4432–4439, 2009.

[13] S. Arif, F. Moore, K. Marks et al., "Peripheral and islet interleukin-17 pathway activation characterizes human autoimmune diabetes and promotes cytokine-mediated β-cell death," *Diabetes*, vol. 60, no. 8, pp. 2112–2119, 2011.

[14] P. Kumar, K. Natarajan, and N. Shanmugam, "High glucose driven expression of pro-inflammatory cytokine and chemokine genes in lymphocytes: molecular mechanisms of IL-17 family gene expression," *Cellular Signalling*, vol. 26, no. 3, pp. 528–539, 2014.

[15] C. Zenobia and G. Hajishengallis, "Basic biology and role of interleukin-17 in immunity and inflammation," *Periodontology 2000*, vol. 69, no. 1, pp. 142–159, 2015.

[16] A. Mitani, W. Niedbala, T. Fujimura et al., "Increased expression of interleukin (IL)-35 and IL-17, but not IL-27, in gingival tissues with chronic periodontitis," *Journal of Periodontology*, vol. 86, no. 2, pp. 301–309, 2015.

[17] O. G. Shaker and N. A. Ghallab, "IL-17 and IL-11 GCF levels in aggressive and chronic periodontitis patients: relation to PCR bacterial detection," *Mediators of Inflammation*, vol. 2012, Article ID 174764, 7 pages, 2012.

[18] P. Behfarnia, R. Birang, S. S. Pishva et al., "Expression levels of th-2 and th-17 characteristic genes in healthy tissue versus periodontitis," *Journal of Dentistry (Tehran, Iran)*, vol. 10, no. 1, pp. 23–31, 2013.

[19] H. Wang, Z. Luo, L. Lei et al., "Interaction between oral lichen planus and chronic periodontitis with Th17-associated cytokines in serum," *Inflammation*, vol. 36, no. 3, pp. 696–704, 2013.

[20] R. A. Awang, D. F. Lappin, A. MacPherson et al., "Clinical associations between IL-17 family cytokines and periodontitis and potential differential roles for IL-17A and IL-17E in periodontal immunity," *Inflammation Research*, vol. 63, no. 12, pp. 1001–1012, 2014.

[21] Z. Luo, H. Wang, Y. Wu, Z. Sun, and Y. Wu, "Clinical significance of IL-23 regulating IL-17A and/or IL-17F positive Th17 cells in chronic periodontitis," *Mediators of Inflammation*, vol. 2014, Article ID 627959, 9 pages, 2014.

[22] M. Chitrapriya, S. R. Rao, and V. Lavu, "Interleukin-17 and interleukin-18 levels in different stages of inflammatory periodontal disease," *Journal of Indian Society of Periodontology*, vol. 19, no. 1, pp. 14–17, 2015.

[23] J. J. Yu, M. J. Ruddy, G. C. Wong et al., "An essential role for IL-17 in preventing pathogen-initiated bone destruction: recruitment of neutrophils to inflamed bone requires IL-17 receptor-dependent signals," *Blood*, vol. 109, no. 9, pp. 3794–3802, 2007.

[24] J. J. Yu, M. J. Ruddy, H. R. Conti, K. Boonanantanasarn, and S. L. Gaffen, "The interleukin-17 receptor plays a gender-dependent role in host protection against *Porphyromonas gingivalis*-induced periodontal bone loss," *Infection and Immunity*, vol. 76, no. 9, pp. 4206–4213, 2008.

[25] R. B. Johnson, N. Wood, and F. G. Serio, "Interleukin-11 and IL-17 and the pathogenesis of periodontal disease," *Journal of Periodontology*, vol. 75, no. 1, pp. 37–43, 2004.

[26] K. Takahashi, T. Azuma, H. Motohira, D. F. Kinane, and S. Kitetsu, "The potential role of interleukin-17 in the immunopathology of periodontal disease," *Journal of Clinical Periodontology*, vol. 32, no. 4, pp. 369–374, 2005.

[27] R. Vernal, N. Dutzan, A. Chaparro, J. Puente, M. A. Valenzuela, and J. Gamonal, "Levels of interleukin-17 in gingival crevicular fluid and in supernatants of cellular cultures of gingival tissue from patients with chronic periodontitis," *Journal of Clinical Periodontology*, vol. 32, no. 4, pp. 383–389, 2005.

[28] Y.-D. Park, Y.-S. Kim, Y.-M. Jung et al., "*Porphyromonas gingivalis* lipopolysaccharide regulates interleukin (IL)-17 and IL-23 expression via SIRT1 modulation in human periodontal ligament cells," *Cytokine*, vol. 60, no. 1, pp. 284–293, 2012.

[29] M. Kadkhodazadeh, Z. Baghani, A. R. Ebadian, N. Youssefi, A. R. Mehdizadeh, and N. Azimi, "IL-17 gene polymorphism is associated with chronic periodontitis and peri-implantitis in Iranian patients: a cross-sectional study," *Immunological Investigations*, vol. 42, no. 2, pp. 156–163, 2013.

[30] M. Kadkhodazadeh, A. R. Ebadian, R. Amid, N. Youssefi, and A. R. Mehdizadeh, "Interleukin 17 receptor gene polymorphism in periimplantitis and chronic periodontitis," *Acta Medica Iranica*, vol. 51, no. 6, pp. 353–358, 2013.

[31] J. D. Corrêa, M. F. Madeira, R. G. Resende et al., "Association between polymorphisms in interleukin-17A and -17F genes and chronic periodontal disease," *Mediators of Inflammation*, vol. 2012, Article ID 846052, 9 pages, 2012.

[32] A. M. Saraiva, M. R. M. Alves e Silva, J. D. F. Correia Silva et al., "Evaluation of IL17A expression and of IL17A, IL17F

and IL23R gene polymorphisms in Brazilian individuals with periodontitis," *Human Immunology*, vol. 74, no. 2, pp. 207–214, 2013.

[33] J. M. Zacarias, E. Â. Sippert, P. Y. Tsuneto, J. E. Visentainer, C. d. Silva, and A. M. Sell, "The Influence of interleukin *17A* and *IL17F* polymorphisms on chronic periodontitis disease in Brazilian patients," *Mediators of Inflammation*, vol. 2015, Article ID 147056, 8 pages, 2015.

[34] L. Pácal, J. Varvařovská, Z. Rušavý et al., "Parameters of oxidative stress, DNA damage and DNA repair in type 1 and type 2 diabetes mellitus," *Archives of Physiology and Biochemistry*, vol. 117, no. 4, pp. 222–230, 2011.

[35] P. Borilova Linhartova, J. Vokurka, H. Poskerova, A. Fassmann, and L. Izakovicova Holla, "Haplotype analysis of interleukin-8 gene polymorphisms in chronic and aggressive periodontitis," *Mediators of Inflammation*, vol. 2013, Article ID 342351, 8 pages, 2013.

[36] J. Bártová, P. Bořilová Linhartová, S. Podzimek et al., "The effect of IL-4 gene polymorphisms on cytokine production in patients with chronic periodontitis and in healthy controls," *Mediators of Inflammation*, vol. 2014, Article ID 185757, 11 pages, 2014.

[37] A. Roohi, M. Tabrizi, F. Abbasi et al., "Serum IL-17, IL-23, and TGF-β levels in type 1 and type 2 diabetic patients and age-matched healthy controls," *BioMed Research International*, vol. 2014, Article ID 718946, 7 pages, 2014.

[38] M. A. Eskan, R. Jotwani, T. Abe et al., "The leukocyte integrin antagonist Del-1 inhibits IL-17-mediated inflammatory bone loss," *Nature Immunology*, vol. 13, no. 5, pp. 465–473, 2012.

[39] S. A. Khader, "Restraining IL-17: Del-1 deals the blow," *Nature Immunology*, vol. 13, no. 5, pp. 433–435, 2012.

[40] S. Takahashi, M. Fukuda, A. Mitani et al., "Follicular dendritic cell-secreted protein is decreased in experimental periodontitis concurrently with the increase of interleukin-17 expression and the Rankl/Opg mRNA ratio," *Journal of Periodontal Research*, vol. 49, no. 3, pp. 390–397, 2014.

[41] W.-C. Cheng, F. J. Hughes, and L. S. Taams, "The presence, function and regulation of IL-17 and Th17 cells in periodontitis," *Journal of Clinical Periodontology*, vol. 41, no. 6, pp. 541–549, 2014.

[42] G. B. N. Nordang, M. K. Viken, J. E. Hollis-Moffatt et al., "Association analysis of the interleukin 17A gene in Caucasian rheumatoid arthritis patients from Norway and New Zealand," *Rheumatology*, vol. 48, no. 4, pp. 367–370, 2009.

[43] J. L. Espinoza, A. Takami, K. Nakata et al., "A genetic variant in the IL-17 promoter is functionally associated with acute graft-versus-host disease after unrelated bone marrow transplantation," *PLoS ONE*, vol. 6, no. 10, Article ID e26229, 2011.

[44] X. K. Liu, X. Lin, and S. L. Gaffen, "Crucial role for nuclear factor of activated T cells in T cell receptor-mediated regulation of human interleukin-17," *The Journal of Biological Chemistry*, vol. 279, no. 50, pp. 52762–52771, 2004.

[45] S. Shao, F. He, Y. Yang, G. Yuan, M. Zhang, and X. Yu, "Th17 cells in type 1 diabetes," *Cellular Immunology*, vol. 280, no. 1, pp. 16–21, 2012.

[46] M. A. G. Ururahy, K. S. C. de Souza, Y. M. D. C. Oliveira et al., "Association of polymorphisms in IL6 gene promoter region with type 1 diabetes and increased albumin-to-creatinine ratio," *Diabetes/Metabolism Research and Reviews*, vol. 31, no. 5, pp. 500–506, 2015.

[47] A. E. Altinova, D. Engin, E. Akbay et al., "Association of polymorphisms in the IL-18 and IL-12 genes with susceptibility to type 1 diabetes in Turkish patients," *Journal of Endocrinological Investigation*, vol. 33, no. 7, pp. 451–454, 2010.

[48] M. P. Hadžija, M. Korolija, N. Jemin et al., "Polymorphisms in the IL-18 and IL-12B genes and their association with the clinical outcome in Croatian patients with Type 1 diabetes," *Gene*, vol. 512, no. 2, pp. 477–481, 2013.

[49] U. K. Gürsoy, S. Y. Çiftlikli, E. Könönen, M. Gürsoy, and B. Doğan, "Salivary interleukin-17 and tumor necrosis factor-α in relation to periodontitis and glycemic status in type 2 diabetes mellitus," *Journal of Diabetes*, vol. 7, no. 5, pp. 681–688, 2015.

[50] A. G. Shabgah, E. Fattahi, and F. Z. Shahneh, "Interleukin-17 in human inflammatory diseases," *Postepy Dermatologii i Alergologii*, vol. 31, no. 4, pp. 256–261, 2014.

[51] L. S. Finoti, S. C. T. Corbi, G. Anovazzi et al., "Association between IL8 haplotypes and pathogen levels in chronic periodontitis," *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 32, no. 10, pp. 1333–1340, 2013.

[52] L. Nibali, D. R. Ready, M. Parkar et al., "Gene polymorphisms and the prevalence of key periodontal pathogens," *Journal of Dental Research*, vol. 86, no. 5, pp. 416–420, 2007.

[53] L. Nibali, M. S. Tonetti, D. Ready et al., "Interleukin-6 polymorphisms are associated with pathogenic bacteria in subjects with periodontitis," *Journal of Periodontology*, vol. 79, no. 4, pp. 677–683, 2008.

[54] L. Nibali, N. Donos, S. Farrell et al., "Association between interleukin-6 -174 polymorphism and *Aggregatibacter actinomycetemcomitans* in chronic periodontitis," *Journal of Periodontology*, vol. 81, no. 12, pp. 1814–1819, 2010.

[55] L. Nibali, I. Madden, F. Franch Chillida, L. J. A. Heitz-Mayfield, P. M. Brett, and N. Donos, "IL6 -174 genotype associated with *Aggregatibacter actinomycetemcomitans* in Indians," *Oral Diseases*, vol. 17, no. 2, pp. 232–237, 2011.

[56] L. Nibali, F. D'Aiuto, D. Ready, M. Parkar, R. Yahaya, and N. Donos, "No association between *A. actinomycetemcomitans* or *P. gingivalis* and chronic or aggressive periodontitis diagnosis," *Quintessence International*, vol. 43, no. 3, pp. 247–254, 2012.

[57] S. Schulz, J. M. Stein, W. Altermann et al., "Single nucleotide polymorphisms in interleukin-1gene cluster and subgingival colonization with *Aggregatibacter actinomycetemcomitans* in patients with aggressive periodontitis," *Human Immunology*, vol. 72, no. 10, pp. 940–946, 2011.

[58] N. M. Moutsopoulos, H. M. Kling, N. Angelov et al., "*Porphyromonas gingivalis* promotes Th17 inducing pathways in chronic periodontitis," *Journal of Autoimmunity*, vol. 39, no. 4, pp. 294–303, 2012.

[59] V. V. Krishnan, R. Ravindran, T. Wun, P. A. Luciw, I. H. Khan, and K. Janatpour, "Multiplexed measurements of immunomodulator levels in peripheral blood of healthy subjects: effects of analytical variables based on anticoagulants, age, and gender," *Cytometry Part B: Clinical Cytometry*, vol. 86, no. 6, pp. 426–435, 2014.

[60] S. S. Khan, M. S. Smith, D. Reda et al., "Multiplex bead array assays for detection of soluble cytokines: comparisons of sensitivity and quantitative values among kits from multiple manufacturers," *Cytometry B, Clinical Cytometry*, vol. 61, no. 1, pp. 35–39, 2004.

Conclusions

Current research is increasingly moving towards cross-disciplinary studies with the collection of data on all aspects of complex diseases. It is crucial to consider gene-environment interactions and to use high-quality methodological approaches, including appropriate analytical tools. Multidisciplinary efforts are required to better understand the etiopathogenesis of complex diseases, such as RAS, EARR, dental caries and CP.

There is a need to create an algorithm involving all known variables to determine the risk of a complex disease development in an individual. Himmelstein and Baranzini (2015) suggested that two important derivations would be the translation of this information into a multiscale understanding of pathogenic variants and leveraging existing data to increase the power of existing and future studies through prioritization. Thus, they designed a network with 18 node types: genes, diseases, tissues, pathophysiologies, and 14 molecular signatures database collections and 19 edge types from high-throughput publicly available resources, see Fig. 15.

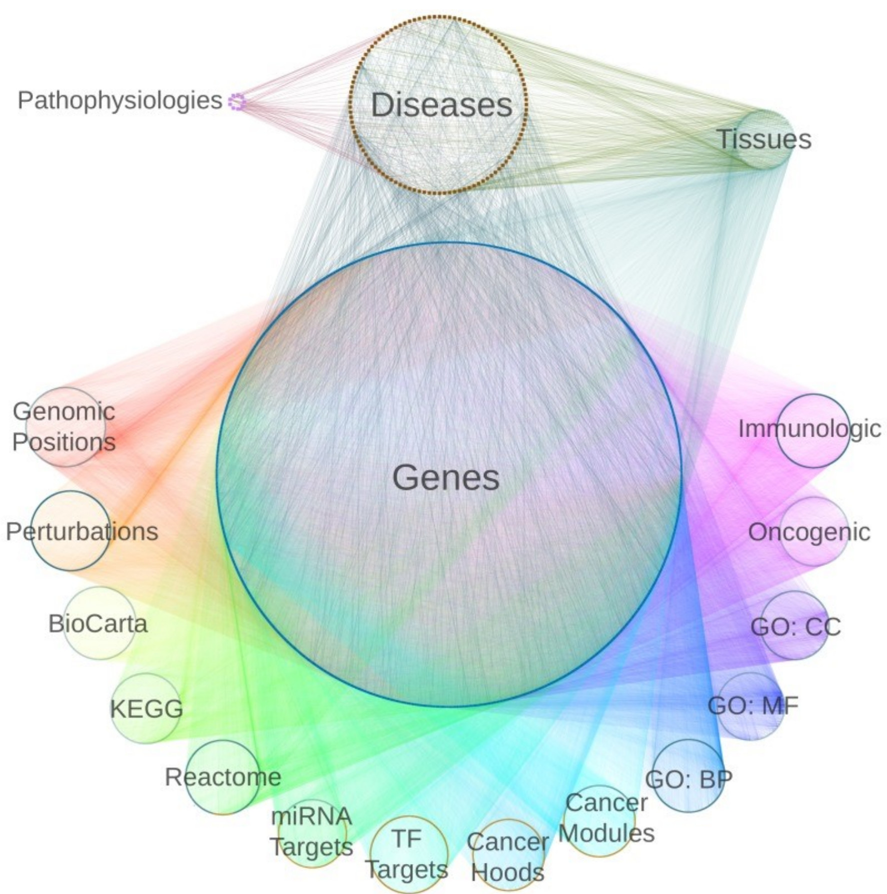


Fig. 15. Heterogeneous network edge prediction effectively prioritized genetic associations and provides a powerful new approach for data integration across multiple domains (Himmelstein and Baranzini, 2015).

A model from GWAS associations was created and the probability of association between each protein-coding gene and each of the well-studied complex diseases was predicted (Himmelstein and Baranzini, 2015). In addition, some other methods for generating multiple risk barcodes of complex diseases using ant colony algorithms are developing (Zhang et al., 2016; Li and Jiang, 2017).

The main goal of immunogenetics is to understand the genetic basis of the immune response and to find the links between gene variability and disease. Although we have shown the relationship of some polymorphisms in genes for immunoregulatory factors to selected diseases of the oral cavity in the Czech population, the complex nature of these diseases requires further research in which genetic analysis will be linked to clinical, microbiological and biochemical examinations. It can be considered important that our statistically non-significant findings have also been published to reduce the positive bias in the scientific literature.
Our immunogenetic research of oral diseases is currently focused on salivary proteins and their genes, especially mannose binding lectin 2 (*MBL2*) with specific functional variants. This pattern recognition receptor of the innate immune system recognizes and binds to pathogenic microorganisms and apoptotic cells leading to lectin pathway complement killing or clearance. MBL heterozygosity could have been advantageous in an evolutionary sense; protection against adverse effects of various infectious diseases and lethal manifestations of atherosclerosis (Eisen and Osthoff, 2014). The original SNaPshot assay and also the algorithm for *MBL2* haplogenotype determination were already designed and introduced. Also, salivary proteins and oral microbiome will be analyzed by advanced molecular biology techniques and all obtained data will be used to design a tool for assessing the risk of development of specific complex oral disease.

References

- Aachoui Y, Sagulenko V, Miao EA, Stacey KJ. Inflammasome-mediated pyroptotic and apoptotic cell death, and defense against infection. Curr Opin Microbiol. 2013;16:319-326.
- Abdel-Moneim A, Bakery HH, Allam G. The potential pathogenic role of IL-17/Th17 cells in both type 1 and type 2 diabetes mellitus. Biomed Pharmacother. 2018;101:287-92.
- Albanidou-Farmaki E, Markopoulos AK, Kalogerakou F, Antoniades DZ. Detection, enumeration and characterization of T helper cells secreting type 1 and type 2 cytokines in patients with recurrent aphthous stomatitis. Tohoku J Exp Med. 2007;212:101-105.
- American Academy of Pediatric Dentistry. Policy on early childhood caries (ECC): classifications, consequences, and preventive strategies 2017;39(6):59-61.
- Anand A, Singh S, Sonkar AA, Husain N, Singh KR, Singh S, Kushwaha JK. Expression of vitamin D receptor and vitamin D status in patients with oral neoplasmsand effect of vitamin D supplementation on quality of life in advanced cancer treatment. Contemp Oncol (Pozn). 2017;21(2):145-151.
- Bartosova M, Svetlak M, Kukletova M, Borilova Linhartova P, Dusek L, Izakovicova Holla L. Emotional stimuli candidates for behavioural intervention in the prevention of early childhood caries: a pilot study. BMC Oral Health. 2019;19(1):33. DOI: 10.1186/s12903-019-0718-4.
- Bartova J, Borilova Linhartova P, Podzimek S, Janatova T, Svobodova K, Fassmann A, Duskova J, Belacek J, Izakovicova Holla L. The effect of IL-4 gene polymorphisms on cytokine production in patients with chronic periodontitis and in healthy controls. Mediators Inflamm. 2014;2014:185757. DOI: 10.1155/2014/185757.
- Bazrafshani MR, Hajeer AH, Ollier WE, Thornhill MH. Polymorphisms in the IL-10 and IL-12 gene cluster and risk of developing recurrentaphthous stomatitis. Oral Dis. 2003;9(6):287-91.
- Bidoki AZ, Harsini S, Sadr M, Soltani S, Mohammadzadeh M, Najafi S, Rezaei N. NLRP3 gene polymorphisms in Iranian patients with recurrent aphthous stomatitis. J Oral Pathol Med. 2016;45:136-140.
- Borilova Linhartova P, Vokurka J, Poskerova H, Fassmann A, Izakovicova Holla L. Haplotype analysis of interleukin-8 gene polymorphisms in chronic and aggressive periodontitis. Mediators Inflamm. 2013;2013:342351. DOI: 10.1155/2013/342351.

- Bořilová Linhartová P. Role genetických faktorů v etiopatogenezi vybraných onemocnění dutiny ústní. Role of genetic factors in the etiopathogenesis of selected diseases of the oral cavity. [Dissertation thesis in Czech]. 2014.
- Bořilová Linhartová P, Valová S, Kaštovský J, Poskerová H, Vokurka J, Fassmann A, Izakovicova Holla L. Genová variabilita v imunoregulačních faktorech u pacientů s chronickou parodontitidou a diabetes mellitus. Gene variability in immunoregulatory factors in patients with chronic periodontitis and diabetes mellitus. [Article in Czech]. Ces Stomatol Prakt Zubni Lek. 2015;115(4):89-97.
- Borilova Linhartova P, Kastovsky J, Bartosova M, Musilova K, Zackova L, Kukletova M, Kukla L, Izakovicova Holla L. ACE insertion/deletion polymorphism associated with caries in permanent but not primary dentition in Czech children. Caries Res. 2016;50(2):89-96.
- Borilova Linhartova P, Kastovsky J, Lucanova S, Bartova J, Poskerova H, Vokurka J, Fassmann A, Kankova K, Izakovicova Holla L. Interleukin-17A gene variability in patients with type 1 diabetes mellitus and chronic periodontitis: its correlation with IL-17 levels and the occurrence of periodontopathic bacteria. Mediators Inflamm. 2016;2016:2979846. DOI: 10.1155/2016/2979846.
- Bořilová Linhartová P, Valová S, Izakovičová Hollá L. Vrozená náchylnost k recidivující aftózní stomatitidě. Genetic predisposition to recurrent aphthous stomatitis. [Article in Czech]. Ces Stomatol Prakt Zubni Lek. 2017;117(2):27-34.
- Borilova Linhartova P, Cernochova P, Kastovsky J, Vrankova Z, Sirotkova M, Izakovicova Holla L. Genetic determinants and postorthodontic external apical root resorption in Czech children. Oral Dis. 2017;23(1):29-35.
- Bořilová Linhartová P, Izakovičová Hollá L. Léčiva a lékové formy jako rizikové faktory pro zubní kaz. Drugs and dosage forms as risk factors for dental caries. [Article in Czech]. Ceska Slov Farm. 2017;66(3):103-6.
- Bořilová Linhartová P, Bartošová L, Křenek A, Bartoš L, Dolina J, Marek F, Kala Z, Izakovičová Hollá L. Personalizovaná terapie pacientů s gastroezofageální refluxní chorobou – metodika stanovení genového profilu CYP2C19. Personalized therapy in patients with gastroesophageal reflux disease – metodology of CYP2C19 gene profile´s determination. [Article in Czech]. Gastroent Hepatol. 2018;72(3):1-9.
- Borilova Linhartova P, Janos J, Slezakova S, Bartova J, Petanova J, Kuklinek P, Fassmann A, Dusek L, Izakovicova Holla L. Recurrent aphthous stomatitis and gene

variability in selected interleukins: a case-control study. Eur J Oral Sci. 2018;126(6):485-492.

- Bořilová Linhartová P, Kukletová M, Izakovicova Holla L. Vztah mezi kojením a výskytem závažného kazu raného dětství. Relationship between breastfeeding and severe early childhood caries. [Article in Czech]. Ces Stomatol Prakt Zubni Lek. 2018;118(3):59-67.
- Borilova Linhartova P, Deissova T, Musilova K, Zackova L, Kukletova M, Kukla L, Izakovicova Holla L. Lack of association between ENAM gene polymorphism and dental caries in primary and permanent teeth in Czech children. Clin Oral Investig. 2018a;22(4):1873-7.
- Borilova Linhartova P, Kavrikova D, Tomandlova M, Poskerova H, Rehka V, Dušek L, Izakovicova Holla L. Differences in interleukin-8 plasma levels between diabetic patients and healthy individuals independently on their periodontal status. Int J Mol Sci. 2018b;19(10), pii: E3214. DOI: 10.3390/ijms19103214.
- Bořilová Linhartová P, Bartošová M. Nové metody v primární prevenci zubního kazu u dětí. New strategies for primary prevention of dental caries in children. Masaryk University, Brno, Czech Republic. 2019. ISBN 978-80-210-9203-7. [online] https://is.muni.cz/auth/do/med/el/nove_metody_v_primarni_prevenci_zubniho_kazu_u_deti/Nove_metody_v_primarni_prevenci_zubniho_kazu_u_deti.pdf
- Borilova Linhartova P, Poskerova H, Tomandlova M, Bartova J, Kankova K, Fassmann A, Izakovicova Holla L. Interleukin-1 gene variability and plasma levels in Czech patients with chronic periodontitis and diabetes mellitus. Int J Dent. 2019;2019:6802349. DOI: 10.1155/2019/6802349.
- Bořilová Linhartová P, Fassmann A, Linhartová J, Izakovičová Hollá L. Farmakoterapie recidivující aftózní stomatitidy u pacientů s geneticky podmíněnou sníženou schopností metabolizovat kyselinu listovou – pilotní studie. Pharmacotherapy of recurrent aphthous stomatitis in patients with genetically impaired ability to metabolize folic acid – pilot study. [Article in Czech]. Ces Stomatol Prakt Zubni Lek. 2019. [Article in press].
- Bořilová Linhartová P, Kavříková D, Slezáková S, Kukletová M, Izakovičová Hollá L. Komerční testy pro stanovení rizika rozvoje zubního kazu a onemocnění parodontu. Commercial tests for the determination of dental caries and periodontal disease risk and development. [Article in Czech]. LKS. 2019;29(2):30-37.

- Boyle EA, Li YI, Pritchard JK. An expanded view of complex traits: from polygenic to omnigenic. Cell. 2017;169(7):1177-1186.
- Bruchard M, Rebé C, Derangère V, et al. The receptor NLRP3 is a transcriptional regulator of TH2 differentiation. Nat Immunol. 2015;16:859-870.
- Ceconi C, Francolini G, Olivares A, Comini L, Bachetti T, Ferrari R. Angiotensin-converting enzyme (ACE) inhibitors have different selectivity for bradykinin binding sites of human somatic ACE. Eur J Pharmacol. 2007;577(1-3):1-6.
- Chapple IL, Bouchard P, Cagetti MG, Campus G, Carra MC, Cocco F, Nibali L, Hujoel P, Laine ML, Lingstrom P, Manton DJ, Montero E, Pitts N, Rangé H, Schlueter N, Teughels W, Twetman S, Van Loveren C, Van der Weijden F, Vieira AR, Schulte AG. Interaction of lifestyle, behaviour or systemic diseases with dental caries and periodontaldiseases: consensus report of group 2 of the joint EFP/ORCA workshop on the boundaries between caries and periodontal diseases. J Clin Periodontol. 2017;44 Suppl 18:S39-S51.
- Cirelli T, Finoti LS, Corbi SCT, Anovazzi G, Nepomuceno R, Orrico SRP, Cirelli JA, Mayer MPA, Scarel-Caminaga RM. Absolute quantification of Aggregatibacter actinomycetemcomitans in patients carryinghaplotypes associated with susceptibility to chronic periodontitis: multifacetedevaluation with periodontitis covariants. Pathog Dis. 2017;75(7).
- Cogulu D, Onay H, Ozdemir Y, Aslan GI, Ozkinay F, Eronat C. The role of vitamin D receptor polymorphisms on dental caries. J Clin Pediatr Dent. 2016;40:211-4.
- Corrêa JD, Madeira MF, Resende RG, Correia-Silva Jde F, Gomez RS, de Souza Dda G, Teixeira MM, Queiroz-Junior CM, da Silva TA. Association between polymorphisms in interleukin-17A and -17F genes and chronic periodontal disease. Mediators Inflamm. 2012;2012:846052.
- Costalonga M, Herzberg MC. The oral microbiome and the immunobiology of periodontal disease and caries. Immunol Lett. 2014;162(2 Pt A):22-38.
- Craig J. Complex diseases: Research and applications. Nature Education. 2008;1(1):184.
- da Silva FRP, Pessoa LDS, Vasconcelos ACCG, de Aquino Lima W, Alves EHP, Vasconcelos DFP. Polymorphisms in interleukins 17A and 17F genes and periodontitis: results from a meta-analysis. Mol Biol Rep. 2017 Dec;44(6):443-53.

- Dakovic D, Colic M, Cakic S, Mileusnic I, Hajdukovic Z, Stamatovic N. Salivary interleukin-8 levels in children suffering from type 1 diabetes mellitus. J Clin Pediatr Dent. 2013;37(4):377-80.
- Deng H, Liu F, Pan Y, Jin X, Wang H, Cao J. BsmI, TaqI, ApaI, and FokI polymorphisms in the vitamin D receptor gene and periodontitis: a meta-analysis of 15 studies including 1,338 cases and 1,302 controls. J Clin Periodontol. 2011;38:199-207.
- Diercke K, Kohl A, Lux CJ, Erber R. IL-1β and compressive forces lead to a significant induction of RANKL-expression in primary human cementoblasts. J Orofac Orthop. 2012;73(5):397-412.
- Divaris K. Predicting dental caries outcomes in children: A "risky" concept. J Dent Res. 2016;95(3):248-54.
- Eisen DP, Osthoff M. If there is an evolutionary selection pressure for the high frequency of MBL2 polymorphisms, what is it? Clin Exp Immunol. 2014;176(2):165-71.
- Fernando S, Speicher DJ, Bakr MM, Benton MC, Lea RA, Scuffham PA, Mihala G, Johnson NW. Protocol for assessing maternal, environmental and epigenetic risk factors for dental caries in children. BMC Oral Health. 2015;15:167.
- Gaetti-Jardim E Jr, Wahasugui TC, Tomazinho PH, Marques MM, Nakano V, Avila-Campos MJ. Distribution of biotypes and leukotoxic activity of Aggregatibacteractinomycetemcomitans isolated from Brazilian patients with chronic periodontitis. Braz J Microbiol. 2008;39(4):658-63.
- Guimarães AL, Correia-Silva Jde F, Sá AR, Victória JM, Diniz MG, Costa Fde O, Gomez RS. Investigation of functional gene polymorphisms IL-1beta, IL-6, IL-10 and TNF-alpha in individuals with recurrent aphthous stomatitis. Arch Oral Biol. 2007;52(3):268-72.
- Hajishengallis E, Parsaei Y, Klein MI, Koo H. Advances in the microbial etiology and pathogenesis of early childhood caries. Mol Oral Microbiol. 2017;32(1):24-34.
- Han M, Fang H, Li QL, Cao Y, Xia R, Zhang ZH. Effectiveness of laser therapy in the management of recurrent aphthous stomatitis: A systematic review. Scientifica (Cairo). 2016;2016:9062430.
- Hartsfield JK Jr, Jacob GJ, Morford LA. Heredity, genetics and orthodontics – How much has this research really helped? Semin Orthod. 2017;23(4):336-347.

- Haworth S, Shungin D, van der Tas JT, Vucic S, Medina-Gomez C, Yakimov V, Feenstra B, Shaffer JR, Lee MK, Standl M, Thiering E, Wang C, Bønnelykke K, Waage J, Jessen LE, Nørrisgaard PE, Joro R, Seppälä I, Raitakari O, Dudding T, Grgic O, Ongkosuwito E, Vierola A, Eloranta AM, West NX, Thomas SJ, McNeil DW, Levy SM, Slayton R, Nohr EA, Lehtimäki T, Lakka T, Bisgaard H, Pennell C, Kühnisch J, Marazita ML, Melbye M, Geller F, Rivadeneira F, Wolvius EB, Franks PW, Johansson I, Timpson NJ. Consortium-based genome-wide meta-analysis for childhood dental caries traits. Hum Mol Genet. 2018;27(17):3113-27.
- Heidari Z, Moudi B, Mahmoudzadeh-Sagheb H. Immunomodulatory factors gene polymorphisms in chronic periodontitis: an overview. BMC Oral Health. 2019;19(1):29.
- Himmelstein DS, Baranzini SE. Heterogeneous network edge prediction: a data integration approach to prioritizedisease-associated genes. PLoS Comput Biol. 2015 Jul 9;11(7):e1004259.
- Hitomi Y, Ebisawa M, Tomikawa M, et al. Associations of functional NLRP3 polymorphisms with susceptibility to food-induced anaphylaxis and aspirin-induced asthma. J Allergy Clin Immunol. 2009;124:779-785.
- Hu J, Hou S, Zhu X, Fang J, Zhou Y, Liu Y, Bai L, Kijlstra A, Yang P. Interleukin-10 gene polymorphisms are associated with Behcet's disease but not with Vogt-Koyanagi-Harada syndrome in the Chinese Han population. Mol Vis. 2015;21:589-603.
- Hu XP, Li ZQ, Zhou JY, Yu ZH, Zhang JM, Guo ML. Analysis of the association between polymorphisms in the vitamin D receptor (VDR) gene and dental caries in a Chinese population. Genet Mol Res. 2015;14:11631-8.
- Izakovicova Holla L, Borilova Linhartova P, Lucanova S, Kastovsky J, Musilova K, Bartosova M, Kukletova M, Kukla L, Dusek L. GLUT2 and TAS1R2 polymorphisms and susceptibility to dental caries. Caries Res. 2015;49(4):417-24.
- Izakovicova Holla L, Valova S, Borilova Linhartova P, Bartova J, Petanova J, Kuklinek P, Fassmann A. Association study of interleukin-1 family, interleukin-6, and its receptor gene polymorphisms in patients with recurrent aphthous stomatitis. J Oral Pathol Med. 2017;46(10):1030-1035.
- Izakovicova Holla L, Borilova Linhartova P, Kastovsky J, Bartosova M, Musilova K, Kukla L, Kukletova M. Vitamin D receptor TaqI gene polymorphism and dental caries in Czech children. Caries Res. 2017;51(1):7-11.

- Jo EK, Kim JK, Shin DM, Sasakawa C. Molecular mechanisms regulating NLRP3 inflammasome activation. Cell Mol Immunol. 2016;13:148-159.
- Jurge S, Kuffer R, Scully C, Porter SR. Mucosal disease series. Number VI. Recurrent aphthous stomatitis. Oral Dis. 2006;12(1):1-21.
- Kadkhodazadeh M, Baghani Z, Ebadian AR, Youssefi N, Mehdizadeh AR, Azimi N. IL-17 gene polymorphism is associated with chronic periodontitis and peri-implantitis in Iranian patients: a cross-sectional study. Immunol Invest. 2013a;42(2):156-63.
- Kadkhodazadeh M, Ebadian AR, Amid R, Youssefi N, Mehdizadeh AR. Interleukin 17 receptor gene polymorphism in periimplantitis and chronic periodontitis. Acta Med Iran. 2013b;51(6):353-8.
- Kastovsky J, Borilova Linhartova P, Musilova K, Zackova L, Kukletova M, Kukla L, Izakovicova Holla L. Lack of association between BMP2/DLX3 gene polymorphisms and dental caries in primary and permanent dentitions. Caries Res. 2017;51(6):590-5.
- Kavrikova D, Borilova Linhartova P, Lucanova S, Poskerova H, Fassmann A, Izakovicova Holla L. Chemokine receptor 2 (CXCR2) gene variants and their association with periodontal bacteria in patients with chronic periodontitis. Mediators Inflamm. 2019;2019:2061868.
- Koch W, Latz W, Eichinger M, Ganser C, Schömig A, Kastrati A. Genotyping of the angiotensin I-converting enzyme gene insertion/deletion polymorphism by the TaqMan method. Clin Chem. 2005;51(8):1547-9.
- Koné-Paut I, Sanchez E, LeQuellec A, Manna R, Touitou I. Autoinflammatory gene mutations in Behçet’s disease. Ann Rheum Dis. 2007;66:832-834.
- Kong YY, Zheng JM, Zhang WJ, Jiang QZ, Yang XC, Yu M, Zeng SJ. The relationship between vitamin D receptor gene polymorphism and deciduous tooth decay in Chinese children. BMC Oral Health. 2017;17(1):111.
- Kornman KS, Polverini PJ. Clinical application of genetics to guide prevention and treatment of oral diseases. Clin Genet. 2014;86(1):44-9.
- Koss K, Satsangi J, Fanning GC, Welsh KI, Jewell DP. Cytokine (TNF alpha, LT alpha and IL-10) polymorphisms in inflammatory bowel diseasesand normal controls: differential effects on production and allele frequencies. Genes Immun. 2000;1(3):185-90.

- Krejsek J, Andrýs C, Krčmová I. Imunologie člověka. Human Immunology. 1. ed. Garamon, Hradec Králové, Czech Republic. [Book in Czech]. 2016.
- Lappin DF, Robertson D, Hodge P, Treagus D, Awang RA, Ramage G, Nile CJ. The influence of glycated hemoglobin on the cross susceptibility between type 1diabetes mellitus and periodontal disease. J Periodontol. 2015 Nov;86(11):1249-59.
- Landová H, Daněk Z, Gajdziok J, Vetchý D, Stembírek J. Orální mukóza a léčba rekurentní aftózní stomatitidy. Oral mucosa and therapy of recurrent aphthous stomatitis. [Article in Czech]. Ceska Slov Farm. 2013;62(1):12-8.
- Lenčová E, Broukal Z. Prevalence zubního kazu u českých předškolních dětí. Caries experience in Czech preschool children. [Article in Czech]. Ces Stomatol Prakt Zubni Lek. 2012;112(6):168-72.
- Li RZ, Ma Xn, Hu XF, Kang SX, Chen SK, Cianflone K, Lu HL. Elevated visfatin levels in obese children are related to proinflammatory factors. J Pediatr Endocrinol Metab. 2013;26(1-2):111-8.
- Li X, Jiang W. Method for generating multiple risky barcodes of complex diseases using ant colony algorithm. Theor Biol Med Model. 2017;14(1):4.
- Lin R. Crosstalk between vitamin D metabolism, VDR signalling, and innate immunity. Biomed Res Int. 2016;2016:1375858.
- Linhartová P, Černochová P, Izakovičová Hollá L. IL1 gene polymorphisms in relation to external apical root resorption concurrent with orthodontia. Oral Dis. 2013;19(3):262-270.
- Lips A, Antunes LS, Antunes LA, Pintor AVB, Santos DABD, Bachinski R, Küchler EC, Alves GG. Salivary protein polymorphisms and risk of dental caries: a systematic review. Braz Oral Res. 2017;31:e41.
- Liu C, Feng X, Li Q, Wang Y, Li Q, Hua M. Adiponectin, TNF-α and inflammatory cytokines and risk of type 2 diabetes: A systematic review and meta-analysis. Cytokine. 2016;86:100-9.
- Mangia A, Santoro R, Piattelli M, Pazienza V, Grifa G, Iacobellis A, Andriulli A. IL-10 haplotypes as possible predictors of spontaneous clearance of HCV infection. Cytokine. 2004 Feb 7;25(3):103-9.
- Mantegazza C, Paglia M, Angiero F, Crippa R. Oral manifestations of gastrointestinal diseases in children. Part 4: Coeliac disease. Eur J Paediatr Dent. 2016;17(4):332-334.

- Martelli FS, Martelli M, Rosati C, Fanti E. Vitamin D: relevance in dental practice. Clin Cases Miner Bone Metab. 2014;11:15-9.
- Mays JW, Sarmadi M, Moutsopoulos NM. Oral manifestations of systemic autoimmune and inflammatory diseases: diagnosis and clinical management. J Evid Based Dent Pract. 2012;12(3 Suppl):265-82.
- Mendoza-Pinto C, García-Carrasco M, Jiménez-Hernández M, Jiménez Hernández C, Riebeling-Navarro C, Nava Zavala A, Vera Recabarren M, Espinosa G, Jara Quezada J, Cervera R. Etiopathogenesis of Behcet´s disease. Autoimmun Res. 2010;9(4):241-245.
- Ministry of Health of the Czech Republic. HEALTH 2020 National strategy for health protection and promotion and disease prevention [online]. http://www.mzcr.cz/verejne/dokumenty/zdravi-2020-narodni-strategie-ochrany-a-podpory-zdravi-a-prevence-nemoci_8690_3016_5.html. Accessed February 14th, 2019.
- Meyle J, Dommisch H, Groeger S, Giacaman RA, Costalonga M, Herzberg M. The innate host response in caries and periodontitis. J Clin Periodontol. 2017;44(12):1215-1225.
- Morrison J, Laurie CC, Marazita ML, Sanders AE, Offenbacher S, Salazar CR, Conomos MP, Thornton T, Jain D, Laurie CA, Kerr KF, Papanicolaou G, Taylor K, Kaste LM, Beck JD, Shaffer JR. Genome-wide association study of dental caries in the Hispanic Communities Health Study/Study of Latinos (HCHS/SOL). Hum Mol Genet. 2016;25(4):807-16.
- Najafi S, Firooze Moqadam I, Mohammadzadeh M, Bidoki AZ, Yousefi H, Farhadi E, Tonekaboni A, Meighani G, Amirzargar AA, Rezaei N. Interleukin-10 gene polymorphisms in recurrent aphthous stomatitis. Immunol Invest. 2014;43(4):405-9.
- Nibali L, Di Iorio A, Onabolu O, Lin GH. Periodontal infectogenomics: systematic review of associations between host geneticvariants and subgingival microbial detection. J Clin Periodontol. 2016;43(11):889-900.
- Novotna M, Podzimek S, Broukal Z, Lencova E, Duskova J. Periodontal diseases and dental caries in children with type 1 diabetes mellitus. Mediators Inflamm. 2015;2015:379626.
- Odegaard JI, Chawla A. Connecting type 1 and type 2 diabetes through innate immunity. Cold Spring Harb Perspect Med. 2012;2(3):a007724.

- Oleksyk TK, Shrestha S, Truelove AL, Goedert JJ, Donfield SM, Phair J, Mehta S, O'Brien SJ, Smith MW. Extended IL10 haplotypes and their association with HIV progression to AIDS. Genes Immun. 2009;10(4):309-22.
- Olszowski T, Adler G, Janiszewska-Olszowska J, Safranow K, Chlubek D: DD Genotype of ACE I/D polymorphism might confer protection against dental caries in Polish children. Caries Res 2015;49:390-3.
- Özen B, Van Strijp AJ, Özer L, Olmus H, Genc A, Cehreli SB. Evaluation of possible associated factors for early childhood caries and severe early childhood caries: a multicenter cross-sectional survey. J Clin Pediatr Dent. 2016;40(2):118-23.
- Poskerová H, Bořilová Linhartová P, Cvanová M, Izakovičová Hollá L. Vliv onemocnení diabetes mellitus na stav chrupu a parodontu. Influence of diabetes mellitus on dental condition and periodontal status. [Article in Czech]. Ces Stomatol Prakt Zubni Lek. 2018;118(1):3-12.
- Poskerová H, Bořilová Linhartová P, Izakovičová Hollá L. Onemocnění dutiny ústní u pacientů s diabetem. Oral diseases in diabetic patients. [Article in Czech]. Vnitr Lek. 2019. [Article in press].
- Pereira S, Lavado N, Nogueira L, Lopez M, Abreu J, Silva H. Polymorphisms of genes encoding P2X7R, IL-1B, OPG and RANK in orthodontic-inducedapical root resorption. Oral Dis. 2014;20(7):659-67.
- Reuss E, Fimmers R, Kruger A, Becker C, Rittner C, Höhler T. Differential regulation of interleukin-10 production by genetic and environmental factors – a twin study. Genes Immun. 2002;3(7):407-13.
- Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I converting enzyme gene accounting for half the variance of serum enzyme levels. J Clin Invest. 1990;86:1343-6.
- Rittling SR, Singh R. Osteopontin in immune-mediated diseases. J Dent Res. 2015;94(12):1638-45.
- Saraiva AM, Alves e Silva MR, Correia Silva Jde F, da Costa JE, Gollob KJ, Dutra WO, Moreira PR. Evaluation of IL17A expression and of IL17A, IL17F and IL23R gene polymorphisms in Brazilian individuals with periodontitis. Hum Immunol. 2013;74(2):207-14.

- Scarel-Caminaga RM, Curtis KM, Renzi R, Sogumo PM, Anovazzi G, Viana AC, Kim YJ, Orrico SR, Cirelli JA. Variation in the CXCR1 gene (IL8RA) is not associated with susceptibility to chronicperiodontitis. J Negat Results Biomed. 2011;10:14.
- Seo JY, Park YJ, Yi YA, Hwang JY, Lee IB, Cho BH, Son HH, Seo DG. Epigenetics: general characteristics and implications for oral health. Restor Dent Endod. 2015;40(1):14-22.
- Senneby A, Mejàre I, Sahlin NE, Svensäter G, Rohlin M. Diagnostic accuracy of different caries risk assessment methods. a systematic review. J Dent. 2015;43(12):1385-93.
- Shaffer JR, Wang X, Feingold E, Lee M, Begum F, Weeks DE, Cuenco KT, Barmada MM, Wendell SK, Crosslin DR, Laurie CC, Doheny KF, Pugh EW, Zhang Q, Feenstra B, Geller F, Boyd HA, Zhang H, Melbye M, Murray JC, Weyant RJ, Crout R, McNeil DW, Levy SM, Slayton RL, Willing MC, Broffitt B, Vieira AR, Marazita ML. Genome-wide association scan for childhood caries implicates novel genes. J Dent Res. 2011;90(12):1457-62.
- Sharab LY, Morford LA, Dempsey J, Falcão-Alencar G, Mason A, Jacobson E, Kluemper GT, Macri JV, Hartsfield JK Jr. Genetic and treatment-related risk factors associated with external apical root resorption (EARR) concurrent with orthodontia. Orthod Craniofac Res. 2015;18 Suppl 1:71-82.
- Silva N, Abusleme L, Bravo D, Dutzan N, Garcia-Sesnich J, Vernal R, Hernández M, Gamonal J. Host response mechanisms in periodontal diseases. J Appl Oral Sci. 2015;23(3):329-55.
- Škach M, Švejda J, Liška K. Onemocnění ústní sliznice: patologie, terapie a diferenční diagnostika onemocnění ústní sliznice. Praha, Avicenum, Czech Republic. [Book in Czech]. 1975.
- Ślebioda Z, Szponar E, Dorocka-Bobkowska B. Vitamin D and its relevance in the etiopathogenesis of oral cavity diseases. Arch Immunol Ther Exp (Warsz). 2016;64(5):385-97.
- Slezakova S, Borilova Linhartova P, Masopustova L, Bartova J, Petanova J, Kuklinek P, Fassmann A, Dusek L, Izakovicova Holla L. Association of the NOD-like receptor 3 (NLRP3) gene variability with recurrent aphthous stomatitis in the Czech population. J Oral Pathol Med. 2018;47(4):434-439.

- Sonnenschein SK, Meyle J. Local inflammatory reactions in patients with diabetes and periodontitis. Periodontol 2000. 2015;69(1):221-54.
- Stanko P, Izakovicova Holla L. Bidirectional association between diabetes mellitus and inflammatory periodontaldisease. A review. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2014;158(1):35-8.
- Stokes L, Fuller SJ, Sluyter R, Skarratt KK, Gu BJ, Wiley JS. Two haplotypes of the P2X(7) receptor containing the Ala-348 to Thr polymorphism exhibit a gain-of-function effect and enhanced interleukin-1beta secretion. FASEB J. 2010;24(8):2916-27.
- Sun C, Chu J, Singh S, Salter RD. Identification and characterization of a novel variant of the human P2X(7) receptor resulting in gain of function. Purinergic Signal. 2010;6(1):31-45.
- Sun M, Fu SM, Dong GY, Wu D, Wang GX, Wu Y. Inflammatory factors gene polymorphism in recurrent oral ulceration. J Oral Pathol Med. 2013;42(7):528-34.
- Tiret L, Rigat B, Visvikis S, Breda C, Corvol P, Cambien F, Soubrier F. Evidence, from combined segregation and linkage analysis, that a variant of the angiotensin I-converting enzyme (ACE) gene controls plasma ACE levels. Am J Hum Genet. 1992;51(1):197-205.
- Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ, Hutchinson IV. An investigation of polymorphism in the interleukin-10 gene promoter. Eur J Immunogenet. 1997 Feb;24(1):1-8.
- Viana AC, Kim YJ, Curtis KM, Renzi R, Orrico SR, Cirelli JA, Scarel-Caminaga RM. Association of haplotypes in the CXCR2 gene with periodontitis in a Brazilian population. DNA Cell Biol. 2010;29(4):191-200.
- Vieira AR, Modesto A, Marazita ML. Caries: review of human genetics research. Caries Res. 2014;48(5):491-506.
- Volckova M, Borilova Linhartova P, Trefna T, Vlazny J, Musilova K, Kukletova M, Kukla L, Izakovicova Holla L. Lack of association between lactotransferrin polymorphism and dental caries. Caries Res. 2014;48(1):39-44.
- Wang X, Shaffer JR, Weyant RJ, Cuenco KT, DeSensi RS, Crout R, McNeil DW, Marazita ML. Genes and their effects on dental caries may differ between primary and permanent dentitions. Caries Res. 2010;44(3):277-84.
- Yan LJ. Pathogenesis of chronic hyperglycemia: from reductive stress to oxidative stress. J Diabetes Res. 2014;2014:137919.

- Yüksel Ş, Eren E, Hatemi G, et al. Novel NLRP3/cryopyrin mutations and pro-inflammatory cytokine profiles in Behçet's syndrome patients. Int Immunol. 2014;26:71-81.
- Zacarias JM, Sippert EÂ, Tsuneto PY, Visentainer JE, de Oliveira e Silva C, Sell AM. The influence of interleukin 17a and IL17f polymorphisms on chronic periodontitis disease in Brazilian patients. Mediators Inflamm. 2015;2015:147056.
- Zhang Q, Li J, Xue H, Kong L, Wang Y. Network-based methods for identifying critical pathways of complex diseases: a survey. Mol Biosyst. 2016;12(4):1082-9.

Figures

Fig. 1. Interactions between internal and external factors in the etiopathogenesis of recurrent aphthous stomatitis (RAS).

Fig. 2. An overview of proteins or groups of proteins encoded by genes that are considered as candidate for recurrent aphthous stomatitis (RAS) and whose variability has been previously studied.

Fig. 3. Classification of selected interleukins previously studied in the context of genetic predispositions to RAS.

Fig. 4. Predicted function of MTHFR enzyme by haplogenotype composed from *MTHFR* rs1801133 and rs1801131 variants. Definition of phenotype of studied patients with RAS and their final subjective evaluation of therapy.

Fig. 5. Number of days of sowing aphthae during therapy.

Fig. 6. 25-OH D_3 serum levels in patients suffered by RAS during the therapy.

Fig. 7. Suggested role of the *NLRP3* rs4612666 SNP in the pathogenesis of RAS.

Fig. 8. Genetic associations to EARR within the ATP/P2RX7/IL-1β and RANK/RANKL/OPG signaling pathways.

Fig. 9. Graphical representation of the incidence of dental caries in primary dentition in children with sECC.

Fig. 10. Stimuli that could be included in future extensive motivation material in an attempt to affect the preventive behaviour of infant's mothers.

Fig. 11. Factors involved in the host susceptibility to the development of dental caries.

Fig. 12. Comparison of the prevalence and severity of periodontitis in diabetic patients and in the HC.

Fig. 13. Comparison of subgingival microflora in diabetic patients and in controls.

Fig. 14. Cytokines and periodontal disease.

Fig. 15. Heterogeneous network edge prediction effectively prioritized genetic associations and provides a powerful new approach for data integration across multiple domains.

Attached files

Attachment 1 Article ”Genetic predisposition to recurrent aphthous stomatitis”
Attachment 2 Article ”Pharmacotherapy of recurrent aphthous stomatitis in patients with genetically impaired ability to metabolize folic acid – pilot study”
Attachment 3 Article ”Personalized therapy in patients with gastroesophageal reflux disease – metodology of *CYP2C19* gene profile's determination”
Attachment 4 Article ”Drugs and dosage forms as risk factors for dental caries”
Attachment 5 Article ”Relationship between breastfeeding and severe early childhood caries”
Attachment 6 Article ”Emotional stimuli candidates for behavioural intervention in the prevention of early childhood caries: a pilot study”
Attachment 7 Article ”Commercial tests for the determination of dental caries and periodontal disease risk and development”
Attachment 8 Article ”Lack of association between *BMP2/DLX3* gene polymorphisms and dental caries in primary and permanent dentitions”
Attachment 9 Article ”Lack of association between *ENAM* gene polymorphism and dental caries in primary and permanent teeth in Czech children”
Attachment 10 Article ”*GLUT2* and *TAS1R2* polymorphisms and susceptibility to dental caries”
Attachment 11 Article ”Influence of diabetes mellitus on dental condition and periodontal status”
Attachment 12 Article ”Oral diseases in diabetic patients”
Attachment 13 Article ”Gene variability in immunoregulatory factors in patients with chronic periodontitis and diabetes mellitus”

Attachment 1 Article ”Genetic predisposition to recurrent aphthous stomatitis”

Přehledový článek
Review

Vrozená náchylnost k recidivující aftózní stomatitidě

(Přehledový článek)

Genetic Predisposition to Recurrent Aphthous Stomatitis

(Review)

ČESKÁ STOMATOLOGIE
ročník 117,
2017, 2,
s. 27–34

Bořilová Linhartová P.[1,2], Valová S.[1,2], Izakovičová Hollá L.[1,2]

[1]Stomatologická klinika LF MU a FN u sv. Anny, Brno
[2]Ústav patologické fyziologie LF MU, Brno

SOUHRN

Předmět sdělení: Recidivující aftózní stomatitida (RAS) je jedním z nejčastějších onemocnění sliznice dutiny ústní, které se projevuje tvorbou bolestivých erozí až ulcerací. Diagnostika RAS je založena na anamnestických údajích a klinickém vzhledu lézí; neexistují žádné laboratorní testy k potvrzení diagnózy. Léčba tohoto onemocnění je pouze symptomatická a málo efektivní. Etiopatogeneze RAS dosud není známa, v literatuře je ale popsána řada rizikových faktorů, které ke vzniku a rozvoji onemocnění mohou přispívat. Kromě lokálního traumatu, potravinových alergenů, mikrobiální dysbiózy, infekčních agens, nutričních faktorů (deficitu vitaminu B12, železa a kyseliny listové), stresu a hormonálních změn, hraje roli i imunologický profil pacienta a také genetické predispozice jedince k této multifaktoriální chorobě. Vliv dědičnosti na vznik, resp. rozvoj onemocnění byl již dříve potvrzen studiemi dvojčat a rodin. Aktuálně jsou publikovány genetické asociační studie zabývající se variabilitou vybraných genů u pacientů s RAS ve srovnání se zdravými kontrolami (tzv. studie kontrol a případů) v různých populacích. Za kandidátní jsou považovány zejména ty geny, které souvisejí s funkcí imunitního systému, s reakcí organismu na oxidační stres, s metabolismem tkání sliznic, vitaminů a minerálních látek.
Cílem těchto analýz bylo nalezení rizikových nebo naopak protektivních variant, a to v genech interleukinů-1 (IL) a antagonisty jejich receptoru (IL-1RN), IL-4, IL-6, IL-10, tumor nekrotizujícího faktoru α (TNFα), NOD-like receptoru 3 (NLRP3), Toll-like receptoru 4 (TLR4), E- a L-selektinů (SEL), angiotenzin konvertujícího enzymu (ACE), v genu pro středomořskou horečku (MEFV), serotoninový transportér (SLC6A4), matrix metaloproteinázu 9 (MMP9), metylentetrahydrofolát reduktázu (MTHFR) a syntázu oxidu dusnatého 2 (NOS2), které mohou v kontextu dalších faktorů ovlivňovat náchylnost jedince k rozvoji onemocnění.
Závěr: V předloženém přehledovém článku jsou shrnuty a diskutovány závěry těchto genetických asociačních studií. Je pravděpodobné, že výzkum RAS na molekulární úrovni by mohl vést k alespoň částečnému pochopení etiopatogeneze tohoto onemocnění, a tím ke zlepšení prevence, diagnostiky a léčby postižených pacientů.
Klíčová slova: *aftózní stomatitida – onemocnění ústní sliznice – genetická studie – genová variabilita – vrozená predispozice*

SUMMARY

Background: Recurrent aphthous stomatitis (RAS), one of the most common diseases of the oral mucosa, is characterized by the formation of painful oral erosions or even ulcers. RAS diagnosis is based on anamnestical data and appearance of lesions; no laboratory tests to confirm the diagnosis are available. Treatment of this condition is only symptomatic and less effective. The disease etiopathogenesis is unknown, but risk factors associated with the origin and development of the disease have been described in the literature. Besides local trauma, food allergens, oral microbial dysbiosis, infectious agents,

ČESKÁ STOMATOLOGIE
ročník 117,
2017, 2,
s. 27–34

nutritious factors (deficiency of B12 vitamin, iron, and folic acid), stress and hormonal changes, the immunological profile of the patient and his/her genetic predispositions to this multifactorial disease play a role. The effect of heredity on the disease origin and development was previously confirmed by studies of twins and families. Genetic variability of the selected genes in patients with RAS compared with healthy controls (case-control study) conducted in different populations have been published. The main candidates for RAS are the genes associated with the immune system, response of the organism to oxidative stress, metabolism of mucosal tissues, vitamins, and minerals.
The aim of these studies was to find risk, or on the contrary protective, gene variants in the interleukin-1 (IL) and its receptor antagonist (IL-1RN), IL-4, IL-6, IL-10, tumor necrosis factor alpha (TNFalpha), NOD-like receptor 3 (NLRP3), Toll-like receptor 4 (TLR4), E- and L-selectin (SEL), angiotensin converting enzym (ACE), gene for Mediterranean fever (MEFV), serotonin transporter (SLC6A4), matrix metalloproteinase 9 (MMP9), methylenetetrahydrofolate reductase (MTHFR) and nitric oxide syntase 2 (NOS2), which may together with other factors influence the individual susceptibility to the disease development. In the present review, we summarize and discuss findings of genetic association studies.
Conclusion: We assume that further research into RAS on the molecular level may lead to better understanding of this disease etiopathogenesis and improve prevention, diagnosis and treatment of the affected patients.
Keywords: *aphthous stomatitis – oral mucosa diseases – genetic study – gene variability – hereditary predisposition*

Čes. Stomat., roč. 117, 2017, č. 2, s. 27–34

ÚVOD

Recidivující aftózní stomatitida (RAS, synonyma: recidivující afty, benigní afty, habituální afty, recidivující benigní aftóza) je chronické zánětlivé onemocnění, které se projevuje opakovaným výsevem erozí až vředů kruhového nebo oválného tvaru na sliznici dutiny ústní. Rozlišujeme tři hlavní klinické formy recidivujících aft: malé afty (Mikuliczovy, MiRAS), velké afty (Suttonovy, MaRAS) a herpetiformní afty (HeRAS) [74]. Ulcerace jsou velmi bolestivé, nejčastěji se vyskytují ve formě tzv. malých aft buď pouze na sliznici dutiny ústní, nebo jsou součástí systémových onemocnění typu Behcetova syndromu (BD), celiakie, Crohnovy choroby, ulcerózní kolitidy, periodické horečky s krční lymfadenopatií, faryngitidy a aftózní stomatitidy (PFAPA) nebo cyklické neutropenie [47, 68].

Diagnostika RAS je založena výhradně na anamnestických údajích a klinickém vzhledu lézí; neexistují žádné laboratorní testy k potvrzení diagnózy [48, 56]. Léčba je v současné době pouze symptomatická a málo efektivní. Dostupnými léčebnými postupy, ať již farmakoterapií [27, 41], nebo laserovou terapií [25], lze pouze snížit bolestivost, frekvenci výskytu nebo závažnost lézí.

Aftózní stomatitida postihuje přibližně 10–25 % celkové populace [19, 23, 62, 64]. K rozvoji onemocnění dochází nejčastěji v období puberty, s přibývajícím věkem frekvence postižení RAS klesá [71]. K onemocnění jsou podle některých studií náchylnější ženy ve srovnání s muži [40]; jiné práce však popisují prakticky stejnou prevalenci RAS u obou pohlaví. Větší riziko vzniku RAS hrozí nekuřákům [49].

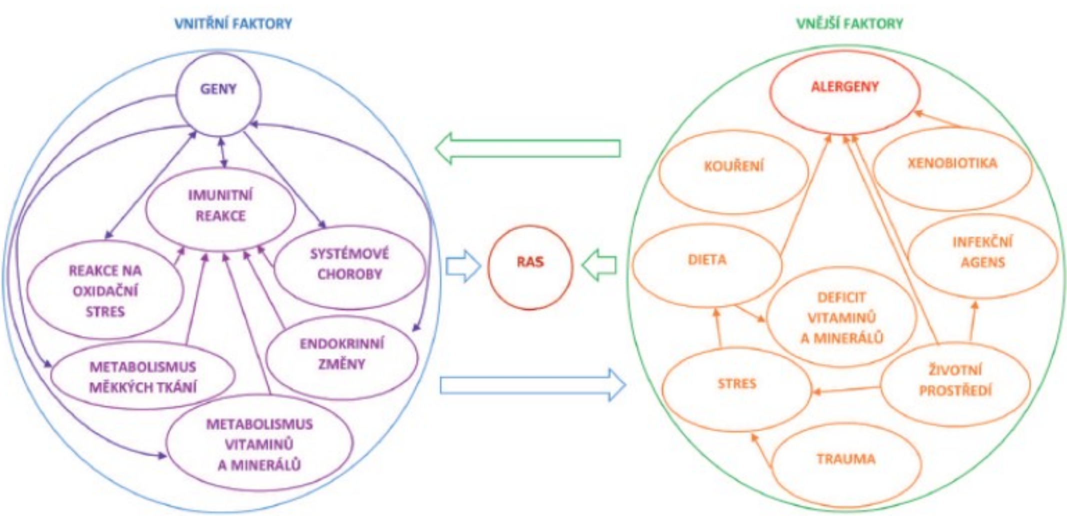
ETIOPATOGENEZE RAS

Etiopatogeneze RAS dosud není zcela objasněna, v literatuře však byla popsána řada rizikových faktorů, které se mohou na vzniku onemocnění podílet. U pacientů s RAS byly oproti zdravým jedincům nalezeny změny v koncentracích minerálů (zinek, železo), vitaminů (kyselina listová, vitamin B12) [45, 58, 73] a proteinů podílejících se na zánětlivých a imunopatologických reakcích (cytokiny a další); byly zaznamenány také změny v expresi genů pro tyto proteiny [4, 9, 15, 17, 18, 43, 44, 51, 59], a to buď lokálně v dutině ústní, a/nebo systémově v krevním oběhu.

Kromě deficitu vitaminů i minerálů existuje řada dalších rizikových faktorů [1], které k rozvoji RAS mohou přispívat. Mezi ně patří lokální trauma sliznice dutiny ústní, potravinové alergeny a mikrobiální dysbióza v dutině ústní [39], přítomnost infekčního agens, hormonální změny a stres [21, 22]. Zásadní roli hraje imunologický profil; dysregulace imunitní odpovědi na vnější podněty může být důsledkem užívání imunosupresiv a jiných xenobiotik, vlivem systémového onemocnění jedince nebo jeho vrozenými predispozicemi [40, 66, 75].

Na obrázku 1 je nastíněna role vybraných vnitřních a vnějších faktorů v etiopatogenezi RAS, a ta-

ČESKÁ STOMATOLOGIE
ročník 117,
2017, 2,
s. 27–34



Obr. 1 Interakce vnitřních a vnějšních faktorů v etiopatogenezi recidivující aftózní stomatitidy (RAS)

ké jejich vzájemné interakce. Faktory exogenního původu mohou ovlivňovat nejenom expresi genů a indukovat vznik jejich mutací, ale mají vliv i na funkci a stav celého organismu. Na druhou stranu vrozené vlastnosti predisponují jedince k určitému typu chování a jednání, např. schopnosti vyvarovat se expozici negativním vlivům vnějšího prostředí. Genetické determinanty hrají roli v regulaci a funkci imunitního a endokrinního systému, k náchylnosti k rozvoji systémových chorob a odolnosti vůči oxidačnímu stresu, jsou také určujícími faktory rozdílů v metabolismu měkkých tkání, vitaminů a minerálů a dalších látek.

VROZENÁ NÁCHYLNOST K RAS

V polovině šedesátých let minulého století byla poprvé popsána role genetických dispozic jedince k rozvoji RAS. Na základě pozorování výskytu RAS v rodinách byl navržen pro tuto chorobu autozomálně recesivní nebo polygenní model dědičnosti [50, 69]. Úloha genetických faktorů v etiopatogenezi onemocnění byla potvrzena následnými asociačními studiemi na rodinách a u dvojčat s RAS [40]. U dítěte s oběma nemocnými rodiči pravděpodobnost, že onemocní, dosahuje až 90 %, zatímco u dětí se zdravými rodiči je jen kolem 20 % [70]. Z výsledků studie 29 rodin vyplývá, že náchylnost k RAS se dědí v souladu s konkrétními haplotypy (kombinované varianty více polymorfních úseků na určitém genu) pro lidské leukocytární antigeny (HLA) [5].

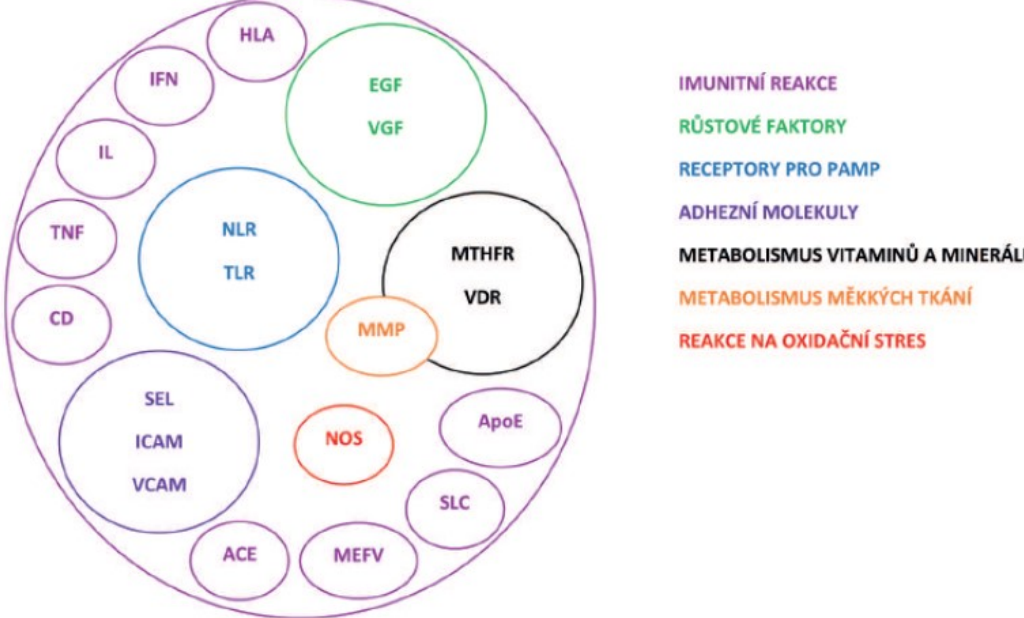
GENETICKÉ ASOCIAČNÍ STUDIE KONTROL A PŘÍPADŮ

Studie kontrol a případů (case-control study), při kterých jsou porovnávány skupiny pacientů s konkrétním onemocněním s adekvátní skupinou (podobný průměrný věk, zastoupení pohlaví, stejná populace apod.) zdravých osob, jsou vedle celogenomových studií (GWAS, genome-wide association study) a analýz rodin (family based study) jednou z variant genetických asociačních studií. U studie kontrol a případů jsou vybrány „kandidátní geny“ pro dané onemocnění; jejich variabilita je zkoumána a výsledky jsou statisticky zpracovány (ideálně pomocí multivariační analýzy, při níž jsou zjišťovány vztahy mezi klinickými, genetickými, imunologickými, biochemickými daty a údaji o zevních faktorech). Pro dostatečnou statistickou sílu testu (power of study) jsou nutné poměrně velké soubory osob zahrnutých do studie. Cílem těchto analýz je nalezení rizikových, nebo naopak protektivních genových variant, které mohou, v kontextu dalších faktorů, ovlivňovat náchylnost jedince k danému onemocnění.

KANDIDÁTNÍ GENY PRO RAS

Za kandidátní jsou považovány geny, které kódují proteiny zapojené do imunitních reakcí organismu, geny související s reakcí organismu na oxidační stres, s metabolismem slizničních struktur, vitaminů a minerálních látek, což logicky navazuje na

ČESKÁ STOMATOLOGIE
ročník 117,
2017, 2,
s. 27–34



Obr. 2 Přehled proteinů nebo skupin proteinů kódovaných geny, které jsou považovány za kandidátní pro recidivující aftózní stomatitidu (RAS) a jejichž variabilita byla již studována
ACE – angiotenzin konvertující enzym, ApoE – apolipoprotein E, CD – diferenciační skupina, EGF – epidermální růstový faktor, ICAM – intercelulární adhezní molekula, IFN – interferon, IL – interleukin, HLA – lidský leukocytární antigen, MEFV – gen pro středomořskou horečku, MMP – matrix metaloproteináza, MTHFR – metylentetrahydrofolát reduktáza, NLR – NOD-like receptor, NOS – syntáza oxidu dusného, SEL – selektin, SLC – serotonin, TLR – Toll-like receptor, TNF – tumor nekrotizující faktor, VCAM – vaskulární buněčná adhezní molekula, VDR – receptor pro vitamin D, VEGF – vaskulární endoteliální růstový faktor

etiopatogenetickou představu onemocnění RAS. Na obrázku 2 jsou uvedeny všechny proteiny (popř. nadřazený termín pro danou skupinu proteinů) kódované geny, jejichž variabilita byla v souvislosti s RAS studována. Je tedy zřejmé, že výzkum byl doposud orientován zejména na kandidátní geny podílející se na reakcích imunitního systému organismu.

Kromě genů kódujících **mediátory zánětu a signální molekuly**, jako jsou interleukiny (IL) a jejich receptory nebo antagonisté receptorů, tumor nekrotizující faktory (TNF) a jejich receptor (Fas) [2, 11, 12, 13, 24, 26, 28, 29, 33, 52, 53, 54, 67, 72, 78] a interferon gama (IFN) [72], byly zkoumány geny pro CD (diferenciační skupina) antigeny na povrchu lymfocytů [37, 79], NOD-like (NLR) [14] a Toll-like **receptory** (TLR) [37] **rozpoznávající molekulární vzory asociované s patogeny** (PAMP, pathogen associated molecular pattern). Mnoho prací bylo zaměřeno na hodnocení úlohy vybraných antigenů HLA tříd v etiopatogenezi RAS [3, 20, 42, 55, 57, 61, 77].

Dalšími studovanými geny u pacientů s RAS byly ty, které kódují **růstové faktory** [72]. Jednalo se o epidermální růstový faktor (EGF), který hraje důležitou roli při růstu, proliferaci a diferenciaci mnoha typů buněk a je produkován především submandibulárními slinnými žlázami a Brunnerovou duodenální žlázou. Významný se zdá být také vaskulární endoteliální růstový faktor (VEGF), multifunkční cytokin účastnící se angiogeneze a hojení ran, jehož signifikantně nižší koncentrace byly nalezeny u pacientů s RAS [16, 60].

Z **adhezních molekul** důležitých pro průběh imunitních reakcí (při zánětu, hojení ran, metastázování atd.) byla u pacientů s RAS sledována variabilita v genech pro selektiny (SEL), intercelulární adhezní molekuly (ICAM) a vaskulární buněčné adhezní molekuly (VCAM) [7].

Dále byl studován inzerčně-deleční polymorfismus v genu pro angiotenzin konvertující enzym (ACE) [32], který je spojován především s funkcí renin-angiotenzin aldosteronového systému, ale také s imunitními procesy [63], a variabilita v genu pro středomořskou horečku (MEFV) podílející na regulaci apoptózy a zánětu [30]. Mezi dalšími analyzovanými byl gen pro apolipoprotein E (ApoE) [10], jehož protein je zapojený nejenom v lipidovém

ČESKÁ STOMATOLOGIE
ročník 117,
2017, 2,
s. 27–34

metabolismu a funkcích endotelu, ale i při supresi zánětlivé odpovědi [6], a také gen pro transportér serotoninu (SLC) [38, 46, 76], neurotransmiteru, který je schopen ovlivňovat mj. funkci polymorfonukleárních leukocytů [65].

Dalším kandidátním genem pro RAS je nukleární receptor pro vitamin D (VDR) [12], který váže vitamin D, a následně tak ovlivňuje transkripci mnoha dalších genů zodpovědných za snížení angiogeneze, zvýšení apoptózy a za imunoregulaci. Ke genům zkoumaným v souvislosti s RAS, které mají současně úlohu v **metabolismu vitaminů nebo minerálů**, patří také gen pro metylentetrahydrofolát reduktázu (MTHFR) [10, 31]. Tento enzym katalyzuje přeměnu kyseliny listové na biologicky aktivní foláty, které plní důležitou funkci v syntetické fázi při buněčném dělení. Ve folátovém cyklu působí jako kofaktor metabolických procesů i vitamin B12.

Matrix metaloproteinázy (MMP) jsou zinek-dependentní endonukleázy schopné degradovat matrixové proteiny, čímž se významně zapojují do **metabolismu měkkých tkání**, jako je sliznice dutiny ústní. Variabilita v genech pro MMP (konkrétně v genech pro gelatinázy) a pro tkáňové inhibitory MMP (TIMP), které hrají roli při zánětlivých procesech, byla již u pacientů s RAS sledována [36].

Tab. 1 Přehled genových variant asociovaných s recidivující aftózní stomatitidou (RAS)

Gen	polymorfismus	asociace s RAS	
IL-1α	rs1800587	C alela je riziková [2]	
IL-1β	rs16944 rs1143634	C alela a CC genotypy jsou rizikové [11] TT genotyp je rizikový [28]	C alela je riziková [2], CT genotyp je rizikový [24]
IL-1RN	86 bp repetice intron 2	alela 1 se 4 repeticemi a 11 genotyp jsou rizikové [11]	
IL-4	70 bp repetice intron 3	alela 3 se 3 repeticemi a 33 genotyp jsou protektivní [29]	
IL-6	rs1800795 rs1800796	CC genotyp je rizikový [54], G alela a GG genotyp jsou rizikové [11, 33] G alela a GG genotyp jsou rizikové [33]	GG haplotyp and GG/GG haplogenotyp jsou rizikové [33]
IL-10	rs1800896 rs1800871 rs1800872	GG genotyp je rizikový [28], AG genotyp je rizikový [53] CT genotyp je rizikový, CC genotyp je protektivní [53] AC genotyp je rizikový, CC genotyp je protektivní [53]	ACC haplotyp je protektivní [53]
TNFα	rs1800629 rs1800630	A alela a AA genotyp jsou rizikové [72] C alela a CC genotyp jsou rizikové [72]	AG genotyp je rizikový [24]
NLRP3	rs3806265	T alela a TT genotyp jsou rizikové	C alela a CT genotyp jsou protektivní [14]
TLR4	rs10759931 rs1927911 rs4986790	A alela a AA genotyp jsou rizikové - -	ACA haplotyp je rizikový, GTA haplotyp je protektivní [37]
E-SEL L-SEL	rs5361 rs1805193 rs2205849	A alela, AA a AC genotypy jsou rizikové - -	AAT haplotyp je rizikový [7]
ACE	I/D intron 16	D alela a DD genotyp jsou rizikové [32]	
MEFV	rs61752717 rs28940580 rs28940579 rs3743930	výskyt mutací je rizikový [30]	
SLC6A4	I/D promotor	S (krátká) alela a SS genotyp jsou rizikové [76]	
MMP9	rs17576 rs11697325	- AA genotyp je rizikový	AA haplotyp je rizikový, GA haplotyp je protektivní [36]
MTHFR	rs1801133	T alela a TT genotyp jsou rizikové [31]	
NOS2	rs2297518 rs1060822	G alela a GG genotyp jsou rizikové -	GG/CC haplogenotyp je rizikový [35]

ČESKÁ STOMATOLOGIE
ročník 117,
2017, 2,
s. 27–34

Oxid dusný, jehož biologická dostupnost je snížena při **oxidačním stresu**, je důležitým mediátorem imunitních reakcí a zánětlivé odpovědi organismu, reguluje adhezi buněk k endotelu, inhibuje agregaci krevních destiček a cévní proliferaci buněk hladkého svalstva [8]. Proto je syntáza oxidu dusného (NOS) považována za kandidátní gen pro RAS a v této souvislosti byly její genové varianty analyzovány [34, 35].

Změny ve struktuře genů, ať už se jedná o délku genu (inzerce, delece jednotlivých bazí nebo větších úseků, jako jsou např. repetitivní sekvence), nebo jeho obsah (záměnové mutace, např. jednonukleotidový polymorfismus, SNP, angl. single nucleotide polymorphism), mohou mít vliv na podobu a funkci výsledného proteinu a/nebo na míru jeho exprese. V případě, že v kandidátním genu existuje funkčně významná mutace, a navíc populační frekvence minoritní alely je alespoň pět procent, můžeme považovat danou variantu za vhodnou pro studium genetické podmíněnosti multifaktoriálních nemocí pomocí asociační studie kontrol a případů. Hovoříme o tzv. polymorfismu příslušného genu, a to již tehdy, pokud se méně častá varianta v dané populaci vyskytuje ve frekvenci vyšší než u jednoho procenta populace.

ASOCIACE VARIANT GENŮ S RAS

Od roku 2002 do současnosti bylo publikováno 36 asociačních studií kontrol a případů s RAS. Genetická variabilita byla zkoumána u třinácti různých populací. Jednalo se o populaci tureckou [2, 29, 30, 31, 32, 33, 57, 61, 78, 79], íránskou [14, 52, 53, 54, 55], jordánskou [7, 35, 36, 37, 38], brazilskou [24, 76, 77], čínskou [28, 72], korejskou [10], indickou [46], egyptskou [26] a populaci z USA [11, 12, 13]. U evropské populace byly dosud zveřejněny studie kontrol a případů s RAS ze Spojeného království [34, 42], z Itálie [20], Řecka [3] a z Polska [67]. Jen u některých kandidátních genů a jejich polymorfismů byla popsána asociace s RAS, přehled je uveden v tabulce 1. Varianty genů byly spojeny s rizikem rozvoje RAS nebo naopak, zdravé osoby s konkrétní alelou, genotypem, haplotypem a/nebo haplogenotypem byly před rozvojem daného onemocnění chráněny (protektivní varianta genu).

U pacientů s RAS byla ve srovnání se zdravými kontrolami pozorována vyšší incidence HLA-A33, HLA-B35 a HLA-B81 [77], HLA-B12 [42], HLA-DR7 [20], HLA-DR5, HLA-A24 [61], HLA-DRB1 a HLA-DRB5, a naopak nižší výskyt HLA-B5, HLA-DR4 [3, 20], HLA-DRB3 [55], HLA-DR10, HLA-DR17 a HLA-A30 [61]. Konkrétní alely HLA-DQB1 pak byly asociovány s protektivitou nebo rizikovostí rozvoje RAS [55] (pozn. není uvedeno v tab. 1).

U některých genových variant byly výsledky asociací s RAS kontroverzní; jednalo se především o variabilitu v genech pro IL-1 a antagonistu jeho receptoru (IL-1RN), IL-6, IL-10 a TNFα [2, 11, 12, 13, 24, 28, 33, 53, 54, 67, 72, 78], dále pak o polymorfismy v genech kódujících MTHFR [10, 31] a SLC6A4 [38, 46, 76]. Tyto protichůdné nálezy mohou být důsledkem genetické odlišnosti různých studovaných populací, nedostatečně velkým souborem studovaných pacientů a kontrol a/nebo jiným přístupem při statistické analýze získaných dat.

ZÁVĚR

Dosud nebyla provedena žádná celogenomová studie (GWAS) u pacientů s RAS, proto jsou v předloženém přehledovém článku shrnuty a zvažovány pouze závěry genetických asociačních studií kontrol a případů s RAS. Ačkoliv jsou výsledky těchto studií v různých populacích často nekonzistentní a význam jednotlivých polymorfismů v genech je obecně u multifaktoriálních onemocnění malý, klíčová role imunitního systému a genetických vloh v etiopatogenezi RAS jsou nesporné. Před tím, než bude možné použít získaná data ke zlepšení diagnostiky či léčby pacientů s RAS, je nezbytný další výzkum.

LITERATURA

1. **Akintoye, S. O., Greenberg, M. S.:** Recurrent aphthous stomatitis. Dental Clinics of North America, roč. 58, 2014, č. 2, s. 281–297.
2. **Akman, A., et al.:** Relationship between periodontal findings and specific polymorphisms of interleukin-1α and-1ß in Turkish patients with Behçet's disease. Arch. Dermatol. Res., roč. 300, 2008, č. 1, s. 19–26.
3. **Albanidou-Farmaki, E., et al.:** HLA-A, B, C, and DR antigens in recurrent oral ulcers. Ann. Dentistry, roč. 47, 1988, č. 1, s. 5–8.
4. **Albanidou-Farmaki, E., et al.:** Detection, enumeration and characterization of T helper cells secreting type 1 and type 2 cytokines in patients with recurrent aphthous stomatitis. Tohoku J. experiment. Med., roč. 212, 2007, č. 2, s. 101–105.
5. **Albanidou-Farmaki, E., et al.:** HLA haplotypes in recurrent aphthous stomatitis: a mode of inheritance? Intern. J. Immunogenet., roč. 35, 2008, č. 6, s. 427–432.
6. **Ali, K., et al.:** Apolipoprotein E suppresses the type I inflammatory response in vivo. Circulation Res., roč. 97, 2005, č. 9, s. 922–927.
7. **Alkhateeb, A., et al.:** Association of cell adhesion molecule gene polymorphisms with recurrent aphthous stomatitis. J. Oral Pathol. Med., roč. 42, 2013, č. 10, s. 741–746.
8. **Andrew, P. J., Mayer, B.:** Enzymatic function of nitric oxide synthases. Cardiovascular Res., roč. 43, 1999, č. 3, s. 521–531.
9. **Avci, E., et al.:** Oxidative stress and cellular immunity in patients with recurrent aphthous ulcers. Brazil. J. Med. Biol. Res., roč. 47, 2014, č. 5, s. 355–360.
10. **Baek, J. O., et al.:** Methylenetetrahydrofolate reductase gene

ČESKÁ STOMATOLOGIE
ročník 117, 2017, 2, s. 27–34

C677T mutation and apolipoprotein E gene mutation in recurrent aphthous stomatitis. Korean J. Dermatol., roč. 46, 2008, č. 2, s. 209–215.

11. **Bazrafshani, M. R., et al.:** IL-1B and IL-6 gene polymorphisms encode significant risk for the development of recurrent aphthous stomatitis (RAS). Genes Immunity, roč. 3, 2002, č. 5, s. 302–305.
12. **Bazrafshani, M. R., et al.:** Recurrent aphthous stomatitis and gene polymorphisms for the inflammatory markers TNF-α, TNF-ß and the vitamin D receptor: no association detected. Oral Dis., roč. 8, 2002, č. 6, s. 303–307.
13. **Bazrafshani, M. R., et al.:** Polymorphisms in the IL-10 and IL-12 gene cluster and risk of developing recurrent aphthous stomatitis. Oral Dis., roč. 9, 2003, č. 6, s. 287–291.
14. **Bidoki, A. Z., et al.:** NLRP3 gene polymorphisms in Iranian patients with recurrent aphthous stomatitis. J. Oral Pathol. Med., roč. 45, 2016, č. 2, s. 136–140.
15. **Borra, R. C., et al.:** The Th1/Th2 immune-type response of the recurrent aphthous ulceration analyzed by cDNA microarray. J. Oral Pathol. Med., roč. 33, 2004, č. 3, s. 140–146.
16. **Brozovic, S., et al.:** Salivary levels of vascular endothelial growth factor (VEGF) in recurrent aphthous ulceration. J. Oral Pathol. Med., roč. 31, 2002, č. 2, s. 106–108.
17. **Buno, I. J., et al.:** Elevated levels of interferon gamma, tumor necrosis factor α, interleukins 2, 4, and 5, but not interleukin 10, are present in recurrent aphthous stomatitis. Arch. Dermatol., roč. 134, 1998, č. 7, s. 827–831.
18. **Dalghous, A. M., Freysdottir, J., Fortune, F.:** Expression of cytokines, chemokines, and chemokine receptors in oral ulcers of patients with Behcet's disease (BD) and recurrent aphthous stomatitis is Th1-associated, although Th2-association is also observed in patients with BD. Scand. J. Rheumatol., roč. 35, 2006, č. 6, s. 472–475.
19. **Feng, J., et al.:** Prevalence and distribution of oral mucosal lesions: a cross-sectional study in Shanghai, China. J. Oral Pathol. Med., roč. 44, 2015, č. 7, s. 490–494.
20. **Gallina, G., et al.:** HLA-A, B, C, Dr, MT, and MB antigens in recurrent aphthous stomatitis. Oral Surg. Oral Med. Oral Pathol., roč. 59, 1985, č. 4, s. 364–370.
21. **Gallo, C. de Barros, Mimura, M. A. M., Sugaya, N. N.:** Psychological stress and recurrent aphthous stomatitis. Clinics, roč. 64, 2009, č. 7, s. 645–648.
22. **Gavic, L., et al.:** The role of anxiety, depression, and psychological stress on the clinical status of recurrent aphthous stomatitis and oral lichen planus. J.Oral Pathol. Med., roč. 43, 2014, č. 6, s. 410–417.
23. **Ghanaei, F. M., et al.:** Prevalence of oral mucosal lesions in an adult Iranian population. Iranian Red Crescent Med. J., roč. 15, 2013, č. 7, s. 600–604.
24. **Guimarães, A. L. S., et al.:** Investigation of functional gene polymorphisms IL-1β, IL-6, IL-10 and TNF-α in individuals with recurrent aphthous stomatitis. Arch. Oral Biol., roč. 52, 2007, č. 3, s. 268–272.
25. **Han, M., et al.:** Effectiveness of laser therapy in the management of recurrent aphthous stomatitis: a systematic review. Scientifica, 2016, 2016, 9062430.
26. **Hazzaa, H. H. A., Rashwan, W. A. M., Attia, E. A. S.:** IL-18 gene polymorphisms in aphthous stomatitis vs. Behçet's disease in a cohort of Egyptian patients. J. Oral Pathol. Med., roč. 43, 2014, č. 10, s. 746–753.
27. **Hosová, K.:** Volně prodejné produkty pro léčbu chorob v dutině ústní. Dermatol. praxi, roč. 5, 2011, č. 1, s. 40–42.
28. **Jing, C., Zhang, J. Q.:** Association between interleukin gene polymorphisms and risk of recurrent oral ulceration. Genet. Molecul. Res., roč. 14, 2015, č. 2, s. 6838–6843.
29. **Kalkan, G., et al.:** Association between interleukin 4 gene intron 3 VNTR polymorphism and recurrent aphthous stomatitis in a cohort of Turkish patients. Gene, roč. 527, 2013, č. 1, s. 207–210.
30. **Kalkan, G., et al.:** Association between MEFV gene mutations and recurrent aphthous stomatitis in a cohort of Turkish patients. J. Dermatol., roč. 40, 2013, č. 7, s. 516–521
31. **Kalkan, G., Karakus, N., Yigit, S.:** Association of MTHFR gene C677T mutation with recurrent aphthous stomatitis and number of oral ulcers. Clin. Oral Investigations, roč. 18, 2014, č. 2, s. 437–441.
32. **Karakus, N., et al.:** High association of angiotensin-converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism with recurrent aphthous stomatitis. Arch. Dermatol. Res., roč. 305, 2013, č. 6, s. 513–517.
33. **Karakus, N., et al.:** Effects of interleukin (IL)-6 gene polymorphisms on recurrent aphthous stomatitis. Arch. Dermatol. Res., roč. 306, 2014, č. 2, s. 173–180.
34. **Karasneh, J. A., et al.:** Endothelial nitric oxide synthase gene polymorphisms are not associated with recurrent aphthous stomatitis. Arch. Oral Biol., roč. 54, 2009, č. 6, s. 583–587.
35. **Karasneh, J. A., et al.:** Association between recurrent aphthous stomatitis and inheritance of a single-nucleotide polymorphism of the NOS2 gene encoding inducible nitric oxide synthase. J. Oral Pathol. Med., roč. 40, 2011, č. 9, s. 715–720.
36. **Karasneh, J. A., et al.:** Association of MMP but not TIMP-1 gene polymorphisms with recurrent aphthous stomatitis. Oral Dis., roč. 20, 2014, č. 7, s. 693–699.
37. **Karasneh, J., et al.:** TLR2, TLR4 and CD86 gene polymorphisms in recurrent aphthous stomatitis. J. Oral Pathol. Med, roč. 44, 2015, č. 10, s. 857–863.
38. **Karasneh, J., et al.:** Investigation of SLC6A4 gene polymorphisms in Jordanian RAS patients. Oral Surg. Oral Med. Oral Pathol. Oral Radiol., roč. 120, 2015, č. 3, s. 141–142.
39. **Kim, Y., et al.:** Mucosal and salivary microbiota associated with recurrent aphthous stomatitis. BMC Microbiol., roč. 16, 2016, č. 1, s. 57.
40. **Koybasi, S., et al.:** Recurrent aphthous stomatitis: investigation of possible etiologic factors. Amer. J. Otolaryngol., roč. 27, 2006, č. 4, s. 229–232.
41. **Landová, H., et al.:** Oral mucosa and therapy of recurrent aphthous stomatitis. Ces. Slov. Farm., roč. 62, 2013, č. 1, s. 12–18.
42. **Lehner, T., Welsh, K. I., Batchelor, J. R.:** The relationship of HLA-B and DR phenotypes to Behcet's syndrome, recurrent oral ulceration and the class of immune complexes. Immunology, roč. 47, 1982, č. 4, s. 581–587.
43. **Lewkowicz, N., et al.:** Predominance of production of Th1 type cytokines in recurrent aphthous ulceration. Dental Medical Problems, roč. 42, 2004, s. 655–660.
44. **Lewkowicz, N., et al.:** Expression of Th1/Th2/Th3/Th17-related genes in recurrent aphthous ulcers. Arch. Immunol. Ther. Experiment., roč. 59, 2011, č. 5, s. 399–406.
45. **Lopez-Jornet, P., Camacho-Alonso, F., Martos, N.:** Hematological study of patients with aphthous stomatitis. Intern. J. Dermatol., roč. 53, 2014, č. 2, s. 159–163.
46. **Manchanda, A., Iyengar, A. R., Patil, S.:** Association between serotonin transporter gene polymorphism and recurrent aphthous stomatitis. Dental Res. J., roč. 13, 2016, č. 3, s. 206–210.
47. **Mantegazza, C., et al.:** Oral manifestations of gastrointestinal diseases in children. Part 4: Coeliac disease. Eur. J. Paed. Dentistry, roč. 17, 2016, č. 4, s. 248–250.

ČESKÁ STOMATOLOGIE
ročník 117, 2017, 2, s. 27–34

48. **Mays, J. W., Sarmadi, M., Moutsopoulos, N. M.:** Oral manifestations of systemic autoimmune and inflammatory diseases: diagnosis and clinical management. J. Evidence Based Dental Pract., roč. 12, 2012, č. 3, s. 265–282.
49. **Mccullough, M. J., Abdel-Hafeth, S., Scully, C.:** Recurrent aphthous stomatitis revisited; clinical features, associations, and new association with infant feeding practices? J. Oral Pathol. Med., roč. 36, 2007, č. 10, s. 615–620.
50. **Miller, M. F., et al.:** Inheritance patterns in recurrent aphthous ulcers: twin and pedigree data. Oral Surg. Oral Med. Oral Pathol., roč. 43, 1977, č. 6, s. 886–891.
51. **Miyamoto J. R., et al.:** Immune-expression of HSP27 and IL-10 in recurrent aphthous ulceration. J. Oral Pathol. Med., roč. 37, 2008, č. 8, s. 462–467.
52. **Moqadam, I. F., et al.:** Lack of association between interleukin-12 gene polymorphisms and recurrent aphthous stomatitis. Avicenna J. med. Biotechnol., roč. 8, 2016, č. 4. s. 200–201.
53. **Najafi, S., et al.:** Interleukin-10 gene polymorphisms in recurrent aphthous stomatitis. Immunological Investigations, roč. 43, 2014, č. 4, s. 405–409.
54. **Najafi, S., et al.:** Association study of interleukin-1 family and interleukin-6 gene single nucleotide polymorphisms in recurrent aphthous stomatitis. Intern. J. Immunogenet., roč. 42, 2015, č.. 6. s. 428–431.
55. **Najafi, S., et al.:** HLA-DRB and HLA-DQB allele and haplotype frequencies in Iranian patients with recurrent aphthous stomatitis. Iranian J. Allergy Asthma Immunol., roč. 15, 2016, č. 4, s. 289–295.
56. **Natah, S. S., et al.:** Recurrent aphthous ulcers today: a review of the growing knowledge. Intern. J. Oral Maxillofacial Surg., roč. 33, 2004, č. 3, s. 221–234.
57. **Özdemir, M., et al.:** HLA-B* 51 in patients with recurrent aphthous stomatitis. Acta derma-venereolog., roč. 89, 2009, č. 2, s. 202–203.
58. **Özler, G. S.:** Zinc deficiency in patients with recurrent aphthous stomatitis: a pilot study. J. Laryngol. Otol., roč. 128, 2014, č. 6, s. 531–533.
59. **Ozyurt, K., et al.:** Serum Th1, Th2 and Th17 cytokine profiles and alpha-enolase levels in recurrent aphthous stomatitis. J. Oral Pathol. Med., roč. 43, 2014, č. 9, s. 691–695.
60. **Pammer, J., et al.** Vascular endothelial growth factor is constitutively expressed in normal human salivary glands and is secreted in the saliva of healthy individuals. J. Pathol., roč. 186, 1998, č. 2, s. 186–191.
61. **Pekiner, F. N., et al.:** HLA-A, B (Class I) and HLA-DR, DQ (Class II) antigens in Turkish patients with recurrent aphthous ulceration and Behcet's disease. Medical Principles Practice, roč. 22, 2013, č. 5, s. 464–468.
62. **Pentenero, M., et al.:** The prevalence of oral mucosal lesions in adults from the Turin area. Oral Dis., roč. 14, 2008, č. 4, s. 356–366.
63. **Reddy Gaddam, R., Chambers, S., Bhatia, M.:** ACE and ACE2 in inflammation: a tale of two enzymes. Inflammation & allergy-drug targets (formerly current drug targets-inflammation & allergy), roč. 13, 2014, č. 4, s. 224–234.
64. **Reichart, P. A.:** Oral mucosal lesions in a representative cross-sectional study of aging Germans. Community Dentistry and Oral Epidemiology, roč. 28, 2000, č. 5, s. 390–398.
65. **Schuff-Werner, P., et al.:** Serotonin acts as a radical scavenger and is oxidized to a dimer during the respiratory burst of human mononuclear and polymorphonuclear phagocytes. Eur. J. Clin. Investigation, roč. 25, 1995, č. 7, s. 477–484.
66. **Ślebioda, Z., Szponar, E., Kowalska, A.:** Etiopathogenesis of recurrent aphthous stomatitis and the role of immunologic aspects: literature review. Archivum Immunologiae et Therapiae Experimentalis, roč. 62, 2014, č. 3, s. 205–215.
67. **Ślebioda, Z., et al.:** Clinical phenotype of recurrent aphthous stomatitis and interleukin-1β genotype in a Polish cohort of patients. J. Oral Pathol. Med., 2016.
68. **Scully, C., Hodgson, T., Lachmann, H.:** Auto-inflammatory syndromes and oral health. Oral Dis., roč. 14, 2008, č. 8, s. 690–699.
69. **Ship, I. I.:** Inheritance of aphthous ulcers of the mouth. J. Dental Res., roč. 44, 1965, č. 5, s. 837–844.
70. **Ship, I. I.:** Epidemiologic aspects of recurrent aphthous ulcerations. Oral Surg. oral Med. Oral Pathol., roč. 33, 1972, č. 3, s. 400–406.
71. **Ship, J. A., et al.:** Recurrent aphthous stomatitis. Quintessence inter., roč. 31, 2000, č. 2, s. 95–112.
72. **Sun, M., et al.:** Inflammatory factors gene polymorphism in recurrent oral ulceration. J. Oral Pathol. Med., roč. 42, 2013, č. 7, s. 528–534.
73. **Sun, A., et al.:** Significant association of deficiencies of hemoglobin, iron, vitamin B12, and folic acid and high homocysteine level with recurrent aphthous stomatitis. J. Oral Pathol. Med., roč. 44, 2015, č. 4, s. 300–305.
74. **Tappuni, A. R., et al.:** Clinical assessment of disease severity in recurrent aphthous stomatitis. J. Oral Pathol. Med., roč. 42, 2013, č. 8, s. 635–641.
75. **Theodoropoulou, K., Vanoni, F., Hofer, M.:** Periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis (PFAPA) syndrome: a review of the pathogenesis. Current Rheumatol. Reports, roč. 18, 2016, č. 4, s. 1–7.
76. **Victoria, J. M. N., et al.:** Serotonin transporter gene polymorphism (5-HTTLPR) in patients with recurrent aphthous stomatitis. J.Oral Pathol. Med., roč. 34, 2005, č. 8. s. 494–497.
77. **Wilhelmsen, N. S. W., et al.:** Correlation between histocompatibility antigens and recurrent aphthous stomatitis in the Brazilian population. Brazilian J. Otorhinolaryngol., roč. 75, 2009, č. 3, s. 426–431.
78. **Yakar, T., et al.:** The relationship of recurrent aphthous stomatitis and *Helicobacter pylori*, cytokine gene polymorphism and cobalamin. Turkish J. Gastroenterol., roč. 26, 2015, č. 4. s. 304–308.
79. **Yigit, S., et al.:** Investigation of CD40 gene rs4810485 and rs1883832 mutations in patients with recurrent aphthous stomatitis. Arch. Oral Biol., roč. 74, 2017, s. 51–54.

Článek vznikl s podporou projektu Specifický výzkum MUNI/A/0948/2016 a grantů AZV 15-29336A a GACR GB14-37368G.

Mgr. Petra Bořilová Linhartová, Ph.D.
Ústav patologické fyziologie
LF MU
Kamenice 5
625 00 Brno
e-mail: plinhart@med.muni.cz

Attachment 2 Article ”Pharmacotherapy of recurrent aphthous stomatitis in patients with genetically impaired ability to metabolize folic acid – pilot study”

V Praze 2. března 2019

Korespondující autor
odbor. as. RNDr. Petra Bořilová Linhartová, Ph.D,
Stomatologická klinika a Ústav patologické fyziologie
Kamenice 5, 62 500 Brno

Potvrzení o převzetí článku k publikaci

Vážená paní asistentko,

Redakce časopisu Česká stomatologie a praktické zubní lékařství (ČSPZL) potvrzuje, že převzala k publikaci odborný článek autorů **Bořilová Linhartová P., Fassmann A., Linhartová J., Izakovičová Hollá L.: Farmakoterapie recidivující aftózní stomatitidy dle individuálního profilu HTHFR.** Článek odpovídá tematickému zájmu našich časopisů a je zařazen v Evidenční knize zaslaných prací pod č. 18/18. Uvedená práce prošla úspěšně recenzním a editorským řízením, takže odpovídá požadavkům Rady pro výzkum, vývoj a inovace Úřadu vlády ČR pro recenzovaná neimpaktovaná periodika. Protokoly o recenzi a průvodní dopis s podpisy jsou uloženy v elektronickém paměťovém médiu redakce pod zn. 1818 RP.

Redakce vzala na vědomí prohlášení o Vaší odborné odpovědnosti za obsah zveřejněných údajů a jejich originálnost v publikovaném článku, který byl schválen všemi uvedenými autory a nikdo z nich není v konfliktu zájmů se studovanou problematikou. Odborné sdělení nebude nabídnuto jinému časopisu či médiu ke zveřejnění. Vydavatel získává otištěním příspěvku výlučné nakladatelské právo k jeho užití.
Distribuce časopisu se uskuteční v jednom obalu společně s časopisem LKS 15. – 18. 4. 2019.

Redakce se těší na spolupráci při Vaší další publikační činnosti.

S pozdravem

doc. MUDr. Jan Veverka, CSc.,
šéfredaktor časopisu Česká stomatologie a praktické zubní lékařství
e-mail: veverkajan20@volny.cz , janveverka20@gmail.com
mobil: +420 607 863 390

FARMAKOTERAPIE RECIDIVUJÍCÍ AFTÓZNÍ STOMATITIDY U PACIENTŮ S GENETICKY PODMÍNĚNOU SNÍŽENOU SCHOPNOSTÍ METABOLIZOVAT KYSELINU LISTOVOU – PILOTNÍ STUDIE

(Původní práce – experimentální klinická studie)

PHARMACOTHERAPY OF RECURRENT APHTHOUS STOMATITIS IN PATIENTS WITH GENETICALLY IMPAIRED ABILITY TO METABOLIZE FOLIC ACID – PILOT STUDY

(Original article – experimental clinical study)

Bořilová Linhartová P.[1, 2], Fassmann A.[1], Linhartová J.[1, 3], Izakovičová Hollá L.[1, 2]

[1]Stomatologická klinika LF MU a FN u sv. Anny, Brno

[2]Ústav patologické fyziologie LF MU, Brno

[3]Lékárna Plumlov, s.r.o., Plumlov

SOUHRN

Úvod a cíl: Při terapii recidivující aftózní stomatitidy (RAS) se doporučuje tzv. Škachova kúra, při které pacienti užívají kyselinu listovou a vitamin B_6. Kyselina listová je v organismu využitelná pouze ve formě aktivního folátu (vitamin B_9); v metabolické přeměně sehrává důležitou úlohu enzym methylentetrahydrofolát reduktáza (MTHFR), jehož funkčnost je zásadně ovlivněna genovými polymorfismy. Cílem práce bylo ověřit předpoklad, že podání aktivní formy kyseliny listové spolu s vitaminy B_6 a D_3 může pozitivně ovlivnit četnost výsevu aft i průběh jejich hojení u pacientů s RAS.

Metody: V rámci pilotní studie jsme klinicky vyšetřili deset pacientů, u nichž jsme analyzovali haplogenotypy v genu *MTHFR*. Podmínky pro zařazení pacientů s RAS byly: věk 18–50 let, česká národnost, geneticky podmíněná snížená schopnost metabolizovat kyselinu listovou (špatný metabolizátor – PM nebo intermediární – IM) a nepřítomnost systémového onemocnění (Crohnova choroba, Behçetova choroba, onemocnění ledvin a další nemoci). Farmakoterapie v upraveném designu dvojitě zaslepené zkřížené studie (cross-over design) probíhala od jara 2018 v délce trvání tří měsíců. Pacientům byl ve třífázovém schématu podáván aktivní folát (glukosaminová sůl 5-methylfolátu), vitaminy B_6 a D_3, po dobu terapie nesměli užívat žádné jiné potravinové doplňky. Vedli si denní záznamy o svém zdravotním stavu a možných rizikových faktorech RAS a byli v měsíčních intervalech klinicky i

laboratorně vyšetřeni. Zaznamenali jsme jak subjektivní hodnocení efektu terapie pacienty, tak i získaná laboratorní data, která byla vyhodnocena statistickým softwarem Statistica v.13.

Výsledky: Do studie jsme zařadili tři muže a sedm žen s RAS ve věku 28 až 47 let; tři z nich byli podle genového profilu *MTHFR* definováni jako PM a sedm jako IM kyseliny listové. Podle subjektivního hodnocení jedna pacientka udala zhoršení stavu, dva pacienti nepozorovali během terapie změnu svého zdravotního stavu a sedm osob bylo spokojeno, neboť u nich došlo k méně častému/žádnému výsevu aft a/nebo doba hojení byla významně kratší. Subjektivní hodnocení terapie pozitivně korelovalo s objektivním klinickým nálezem ($r = 0{,}993$, $p < 0{,}001$). Z laboratorní analýzy vyplynulo, že podání vitaminu D_3 signifikantně zvýšilo sérové koncentrace 25-OH D_3 z nízkých/limitních na optimální hodnoty. Medián koncentrací před zahájením studie byl 54,2 $nmoll^{-1}$, těsně před podáním 68,1 $nmoll^{-1}$ vs. měsíc po podání 96,6 $nmoll^{-1}$ (norma: 50–175 $nmoll^{-1}$), ($p \leq 0{,}01$). Další sledované parametry,çççç jako koncentrace kyseliny listové v séru, homocysteinu, ALT, AST a krevní obraz byly u pacientů ve fyziologickém rozmezí před terapií i po ní.

Závěr: Navržená modifikace Škachovy vitaminové kúry se zdá být vhodná pro léčbu pacientů s RAS, u kterých je genetická predispozice ke snížené funkci enzymu MTHFR, neboť během našeho pozorování došlo u 70 % pacientů k subjektivnímu i objektivnímu zlepšení stavu.

KLÍČOVÁ SLOVA

onemocnění sliznice dutiny ústní, farmakogenetika, metabolismus, kyselina listová, vitamin D

SUMMARY

Introduction, aim: When treating recurrent aphthous stomatitis (RAS), a so-called Skach's therapy, in which patients are administered folic acid and vitamin B_6, is recommended. The human body can process folic acid only in the form of active folate (vitamin B_9); methylenetetrahydrofolate reductase (MTHFR) plays an important role in metabolic transformation, function of this enzyme is fundamentally influenced by gene polymorphisms.

The aim of the study was to verify the assumption that administration of the active form of folic acid together with vitamins B_6 and D_3 can positively affect the frequency of aphthae/ulcers eruption and the course of their healing in patients with RAS.

Methods: In the pilot study, we clinically examined ten patients in whom we analyzed haplogenotypes in the *MTHFR* gene. Conditions for inclusion of patients with RAS were: age 18–50 years, Czech nationality, genetically impaired ability to metabolize folic acid (poor metaboliser – PM or intermediate – IM) and the absence of systemic disease (Crohn's disease, Behçet's disease, kidney disease and other diseases). Pharmacotherapy in a modified double-crossed blind study (cross-over design) ran from spring 2018 and lasted three months. Patients

were treated with active folate (glucosamine salt 5-methylfolate), vitamins B_6 and D_3 in the three-phase regimen, they were not allowed to use any other food supplements during the therapy. They kept daily records of their health status and possible risk factors for RAS and were examined clinically and in the laboratory at monthly intervals. We recorded both the subjective evaluation of the effect of the therapy by patients and the obtained laboratory data, which were evaluated by statistical software Statistica v.13.

Results: The study was comprised of three men and seven women with RAS, aged 28 to 47 years; according to the *MTHFR* gene profile, three were defined as PM and seven as IM of folic acid. Based on the subjective assessment, one patient reported a deterioration, two patients did not observe a change in their health state during therapy, and seven were satisfied because they had a lower or no aphthae eruption and/or healing time was significantly shorter. Subjective evaluation of therapy positively correlated with objective clinical finding ($r = 0.993$, $P < 0.001$). The laboratory analysis showed that administration of vitamin D_3 significantly increased serum concentrations of 25-OH D_3 from low/limit to optimal values, median of concentrations prior to study was 54.2 $nmoll^{-1}$, just before administration 68.1 $nmoll^{-1}$ vs. month after administration 96.6 $nmoll^{-1}$ (norm: 50–175 nmoll-1), ($P \leq 0.01$). The other studied parameters, such as concentration of folic acid in serum, homocysteine, ALT, AST, and blood count, were within the physiological range before and after therapy.

Conclusion: The proposed modification of Skach's vitamin therapy seems to be appropriate for the treatment of patients suffering from RAS with genetic predisposition for reduced MTHFR function as during our observation, 70% of patients experienced subjective and objective improvement of their state.

KEYWORDS

oral mucosa disease, pharmacogenetics, metabolism, folic acid, vitamin D

ÚVOD

Recidivující aftózní stomatitida (RAS) je chronické onemocnění sliznice dutiny ústní s dosud nejasnou etiopatogenezí, které postihuje až 25 % populace [1]. Jsou známy pouze rizikové faktory, mezi které se řadí genetická predispozice [2], imunopatologické reakce a přítomnost infekčního agens, endokrinní i psychické vlivy (stres), nutriční deficience (železo, kyselina listová, vitaminy skupiny B) a lokálně působící faktory (přikusování, poranění při stomatologickém ošetření a jiné). Aftám podobné ulcerace jsou symptomem mnoha systémových onemocnění, např. Crohnovy choroby (CD), Behçetovy choroby (BD), ulcerózní kolitidy, celiakie, neutropenie a dalších.

Kauzální léčba RAS dosud neexistuje, v současnosti máme k dispozici pouze symptomatickou terapii. Jedním z léčebných přístupů je podání tzv. Škachovy vitaminové kúry [3] s různými modifikacemi (délka a opakování terapie). Podávají se při ní preparáty obsahující kyselinu listovou (Acidum folicum 2x denně 1 tbl.), vitaminy B_6 (Pyridoxin 3x denně 1 tbl.) a B_{12} (intramuskulárně 300 µg obden), a to nikoliv kontinuálně, ale intermitentně s pauzami, například ve schématu: třítýdenní kúra – tři týdny pauza – třítýdenní kúra – šest týdnů pauza – třítýdenní kúra.

Na podání Škachovy vitaminové kúry však pozitivně nereagují všichni pacienti s RAS, což může být dáno sníženou schopností jejich organismu metabolizovat některé z jejích složek. Kyselina listová je v organismu využitelná pouze ve formě aktivního folátu (L-5-methylfolát, vitamin B_9); v metabolické přeměně sehrává důležitou úlohu enzym methylentetrahydrofolát reduktáza (MTHFR). Snížené koncentrace 5-methylfolátu jsou pozorovány u pacientů s diabetes mellitus, CD, atrofickou gastritidou, hyperhomocysteinémií a jinými chorobami. Za rizikové faktory spojené s nedostatečnou koncentrací L-methyl folátu je považována obezita i malnutrice, kouření, expozice těžkým kovům a toxinům, užívání alkoholu a některých léčiv (orální kontraceptiva, orální antidiabetika, antikonvulziva, metotrexát) a také polymorfismy v genech kódujících enzymy zapojené v metabolizaci kyseliny listové. Z nich je nejvíce studována variabilita v genu pro methylentetrahydrofolát reduktázu (MTHFR), konkrétně funkční varianty C677T (rs1801133, Ala222Val) a A1298C (rs1801131, Glu429Ala).

Přítomnost minoritní alely T C677T v sekvenci genu *MTHFR* vede při jeho expresi ke vzniku termolabilního enzymu. Také polymorfismus A1298C v genu *MTHFR* byl asociován se sníženou aktivitou enzymu, se změnou distribuce intracelulárního folátu a koncentrací homocysteinu [4]. U heterozygotů C677T a homozygotů AA A1298C (+-/-- haplogenotyp) je aktivita enzymu MTHFR mírně vyšší (přibližně 65%) než u heterozygotů pro obě varianty (přibližně 50%), proto byl A1298C polymorfismus popsán jako faktor přispívající k výslednému fenotypu [5]. Po analýze genotypů obou variant lze jednotlivým kombinacím (haplogenotypům, v tomto případě diplotypům) přiřadit míru ztráty funkce enzymu neboli teoretický fenotyp (tab. 1).

Tab. 1 Předpokládaná funkce enzymu MTHFR podle haplogenotypu C677T a A1298C variant genu *MTHFR*. Definice fenotypu studovaných pacientů s RAS a jejich výsledné subjektivní hodnocení terapie.

SNP		A1298C *MTHFR* (rs1801131) MAF (C) = 34 %			
	genotyp **funkce enzymu MTHFR** **frekvence v EUR populaci**	AA 100 % 43 %	AC 80–100 % 45 %	CC 60 % 12 %	
C677T *MTHFR* (rs1801133) MAF (T)=31 %	CC 100 % 46 %	--/-- 100 % EM	--/+- 80 % EM	--/++ 60 % IM	
	CT 65 % 44 %	+-/-- 65 % IM	+-/+- 50 % IM	+-/++ 30 % PM	
	TT 20–30 % 10 %	++/-- <30 % PM	++/+- <30 % PM	++/++ <10 % PM	**haplogenotyp** **funkce enzymu MTHFR** **předpokládaný fenotyp** **osoby s RAS v naší studii**

+ = minoritní alela; - = majoritní alela; SNP = jednonukleotidový polymorfismus; MAF = frekvence minoritní alely;

EM = normální metabolizátor; IM = intermediární metabolizátor; PM = špatný metabolizátor;

modrá/černá/červená osoba = pozitivní/neutrální/negativní hodnocení terapie subjektem;

pozn.: alelické a genotypové frekvence podle NCBI databáze pro evropskou populaci

Vitamin D_3 (cholekalciferol) je významným imunoregulátorem, neboť má jak protiinfekční a imunopotenciační, tak také imunosupresivní účinky. Vzhledem k tomu, že je u pacientů s RAS i BD často pozorována deficience vitaminu D [6], zdá se mít jeho suplementace potenciál v léčbě i profylaxi těchto stavů [7].

Cílem práce bylo ověřit předpoklad, že podání aktivní formy kyseliny listové spolu s vitaminy B_6 a D_3 může pozitivně ovlivnit četnost výsevu aft i průběh/rychlost jejich hojení u pacientů s RAS, kteří mají sníženou aktivitu enzymu MTHFR.

METODIKA

Studované osoby

Podmínky pro zařazení pacientů s RAS byly: věk 18–50 let, česká národnost, nepřítomnost systémového onemocnění (CD, BD, onemocnění ledvin a dalších chorob) a geneticky podmíněná snížená schopnost metabolizovat kyselinu listovou (špatný metabolizátor – PM nebo intermediární metabolizátor – IM). Pacienti s alergií na laktózu nebyli do studie zařazeni vzhledem k tomu, že podávaný přípravek obsahoval laktózu.

Podepsaný informovaný souhlas byl schválený etickou komisí FNUSA Brno. Pacienti nebyli finančně ani jinak za účast ve studii odměněni.

Klinické vyšetření a laboratorní analýza

Před zařazením do studie byli pacienti detailně vyšetřeni zkušeným zubním lékařem. Nemocní se podrobili i klinickému a laboratornímu imunologickému vyšetření v Ústavu klinické imunologie a alergologie FNUSA v Brně, kde byly sledovány zejména parametry k potvrzení/vyloučení systémových onemocnění, jako je CD, BD, ulcerózní kolitida, celiakie a další, které jsou spojované s tvorbou aftám podobných ulcerací nejenom na sliznici dutiny ústní.

V průběhu studie byly pacientům provedeny odběry žilní krve podle protokolu před zahájením první fáze a po jejím ukončení a po druhé a třetí fázi (celkem čtyři odběry). Ve stejných časech byli pacienti opakovaně vyšetřeni zubním lékařem a byl jim podán individuálně vyráběný léčivý přípravek (IVLP), na začátku druhé fáze i tableta s vitaminem D_3. Po ukončení studie všichni pacienti obdrželi závěrečnou zprávu o výsledcích vyšetření. V laboratoři FNUSA v Brně byly standardními metodami analyzovány následující parametry: krevní obraz + diferenciál, glomerulární filtrace, koncentrace Ca^{2+}, kreatininu, homocysteinu, kyseliny listové, vitaminu B_{12}, ALT, AST a 25-OH D_3.

Izolace DNA a genetická analýza

Před zařazením do studie jsme z 9 ml žilní krve odebrané do zkumavky s EDTA izolovali DNA pacientů standardní izopropanol-fenol-chloroformovou metodou s využitím proteinázy K.

V Ústavu patologické fyziologie LF MU jsme analyzovali polymorfismy C677T (rs1801133) a A1298C (rs1801131) v genu *MTHFR* metodou qPCR s fluorescenčně značenými TaqMan sondami (C___1202883_20 a C____850486_20, ThermoScientific, Grand Island, NY, USA) na přístroji ABI PRISM 7000, Applied Biosystems, USA. Data byla vyhodnocena softwarem SDS version 1.2.3, Applied Biosystems, USA.

Podle dostupné literatury byl určen předpokládaný fenotyp pacienta odpovídající jeho haplogenotypovému profilu *MTHFR*. Osoby s aktivitou enzymu MTHFR 30–65 % jsou považovány za IM a pacienti s nižší funkcí (< 30 % normální aktivity enzymu) byli označeni fenotypem PM.

Výroba IVLP

Celkem jsme v lékárně vyrobili deset sad po třech baleních s 28 tobolkami s bílou sypkou hmotou (840 průhledných tobolek). Každý pacient obdržel na začátku každé fáze jeden šroubovací plastový kelímek popsaný jeho jménem, číslem fáze, datem výroby a spotřeby IVLP a místem výroby s podpisem odpovědné osoby. Celkem 560 tobolek obsahovalo pouze 0,5 g lactosum monohydricum a 280 tobolek obsahovalo 60 mg pyridoxinum hydrochloricum se 400 µg glukosaminové soli 5-methylfolátu a lactosum monohydricum q.s.

Design terapie

Osoby zařazené do naší studie v průběhu tří měsíců v období jara 2018 podstoupily třífázovou terapii, která byla navržena jako dvojitě zaslepená (double-blinded) zkřížená studie (cross-over design) s modifikací. Při standardní zkřížené studii pacient v randomizovaném pořadí dostane postupně obě terapie, mezi nimi je wash-out perioda, která musí být dostatečně dlouhá na to, aby výsledek druhé léčby nebyl ovlivněn užíváním přípravku v první terapii. Modifikace obvyklého designu spočívala v tom, že studie pro všechny pacienty probíhala ve schématu, kdy po kontrolní fázi následovala fáze terapeutická a jako poslední proběhla sledovací fáze.

Neporovnávali jsme přitom dvě terapie, ale období bez užívání vitaminových doplňků a účinek navržené vitaminové kúry a její efekt. Ošetřující zubní lékař ani pacient neměli informaci o tom, v jaké fázi jsou vitaminy v IVLP obsaženy. Každá fáze trvala 28 dní a během ní pacienti každý den užívali jednu tobolku a denně zaznamenávali do jednoduchého papírového archu, že tobolku vzali, zda měli/neměli výsev aft a jejich případnou lokalizaci, přítomnost stresu, jiného onemocnění, kouření, příjem alkoholu (i jeho druh), přítomnost dalších rizikových faktorů (potravin, návštěvy zubního lékaře, mechanické podráždění a jiné), a ženy uváděly i dobu menstruace.

V první (kontrolní) fázi pacienti užívali 0,5 g lactosum monohydricum, na začátku druhé (terapeutické) fáze byl pacientům v ordinaci podán vitamin D_3 s postupným uvolňováním po dobu 12 týdnů (Vitamin D_3 Axonia 30 000 UI/1 tbl. – obvykle předepisováno jako měsíční dávka), a denně užívali tobolku s obsahem 60 mg pyridoxinum hydrochloricum s 400 µg glukosaminové soli 5-methylfolátu a lactosum monohydricum q.s. Před podáním vitaminu D_3 byla laboratorně zkontrolována funkce ledvin a koncentrace Ca^{2+} a kreatininu, neboť hyperkalcémie i onemocnění ledvin jsou podle souhrnu údajů o přípravku (SPC) kontraindikací jeho použití. Ve třetí (sledovací) fázi byl podáván IVLP shodný s první fází.

Statistická analýza

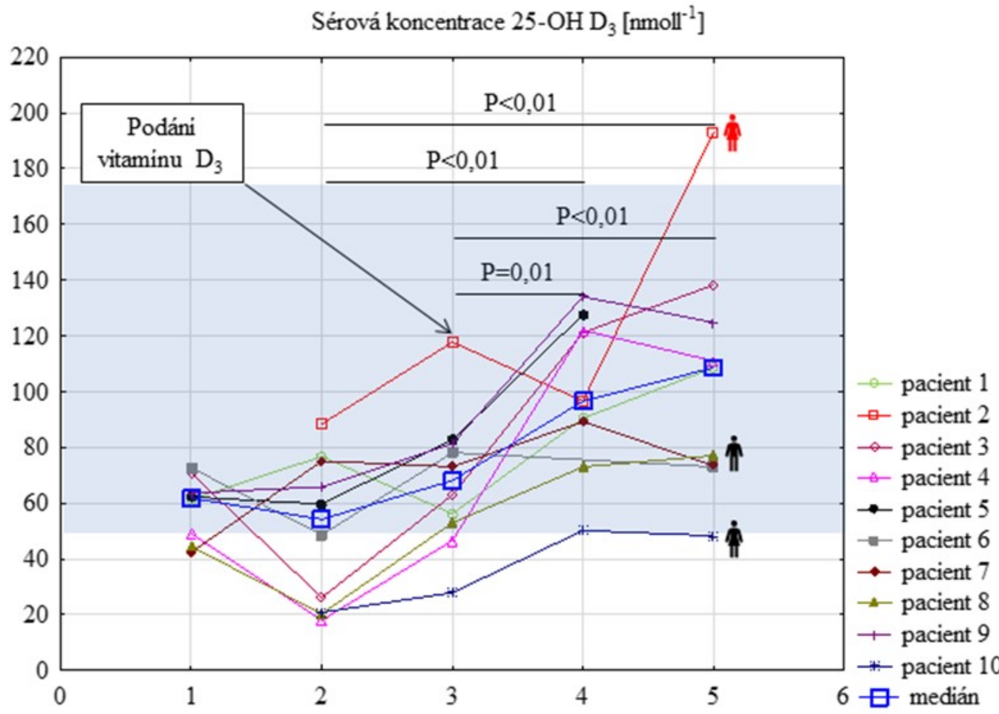
Ke statistickému hodnocení bylo využito zejména neparametrických testů (Wilcoxonův párový test, Spearmanův korelační test). Za hladinu významnosti byla zvolena p-hodnota $\leq$ 0,05. Data byla vyhodnocena statistickým softwarem Statistica v.13.

VÝSLEDKY

Do studie jsme zařadili tři muže a sedm žen s RAS ve věku 28 až 47 let, tři z nich byli podle genového profilu MTHFR definováni jako PM a sedm jako IM kyseliny listové. Tři ženy uvedly, že kouří, a to tři až deset cigaret denně, ostatní pacienti byli nekuřáci. Tři pacienti jsou léčení alergici.

Všech deset osob trpělo podle klinického vyšetření před zahájením terapie na aftyklol typu minor s frekvencí výsevu minimálně jedenkrát za tři měsíce v počtu tří a více afekcí. Celkem 50 % těchto osob před začátkem terapie uvedlo, že ulcerace na sliznici dutiny ústní má prakticky permanentně. U poloviny osob ze souboru docházelo k difuznímu výsevu aft, u dalších se jednalo o různé lokalizace: v oblasti patrových oblouků, v horní a/nebo dolní čelisti vestibulárně, na vnitřní sliznici dolního rtu, v podjazykové krajině a na bukálních sliznicích. Obvyklá doba hojení aft byla u tří osob do deseti dní a u sedmi osob delší než 14 dní. Žádný z pacientů neuváděl přítomnost ulcerací jinde než na sliznici dutiny ústní. Medián věku, kdy se pacientovi poprvé objevila afta, byl 15 let (12–18 let, kvartil 25 % – kvartil 75 %). Dva pacienti (20 % ze souboru) uvedli, že RAS trpí i jejich rodič a/nebo potomek. Jako nejčastější rizikové faktory pacienti zmiňovali infekční onemocnění, psychický stres a mechanické podráždění.

Před zařazením do studie 70 % osob pravidelně užívalo vitaminy skupiny B. Při primárním screeningu u nich byl medián sérové koncentrace 25-OH D_3 61,8 $nmoll^{-1}$ (46,8–67,3 $nmoll^{-1}$); norma je 50–175 $nmoll^{-1}$. Tento odběr však proběhl v různých ročních obdobích, proto nebyl statisticky dále hodnocen. Před první (kontrolní) fází byl medián sérové koncentrace 25-OH D_3 u pacientů 54,2 $nmoll^{-1}$ (20,8–75,1 $nmoll^{-1}$), těsně před podáním vitaminu D_3 68,1 $nmoll^{-1}$ (53,0–81,4 $nmoll^{-1}$). Měsíc po jeho podání byl medián sérové koncentrace 25-OH D_3 96,6 $nmoll^{-1}$ (89,2–122,0 $nmoll^{-1}$) a po 56 dnech 108,6 $nmoll^{-1}$ (73,5–124,8 $nmoll^{-1}$). Po podání vitaminu D_3 se signifikantně zvýšila sérová koncentrace 25-OH D_3 ($p \leq 0,01$, hodnoceno Wilcoxonovým párovým testem), viz graf 1. U pacientky č. 2 byla koncentrace při ukončení terapie nad normu (193,1 $nmoll^{-1}$) a u pacientky č. 10 byla i přes podání vitaminu D_3 diagnostikována snížená koncentrace 25-OH D_3.



Graf 1. Sérové koncentrace 25-OH D_3 u pacientů s RAS před, v průběhu a při ukončení terapie

černá/červená osoba=neutrální/negativní hodnocení terapie subjektem

Pozn. Chybí 4 hodnoty koncentrace v různém čase u různých pacientů z důvodu selhání laboratorní analýzy.

Hodnoceno Wilcoxonovým párovým testem. Stav před zařazením do sledované skupiny pacientů s RAS nebyl do analýzy zahrnut, jelikož stanovení nebylo u pacientů provedeno ve stejném ročním období a výsledky by byly zavádějící.

Modrá oblast znázorňuje normu sérové koncentrace 25-OH D_3 (50-175 $nmoll^{-1}$).

Osa x

1=stav před zařazením do sledované skupiny

2=stav před 1. (kontrolní) fází

3=stav před 2. (terapeutickou) fází, po odběru krve byla podána tableta vitamínu D_3

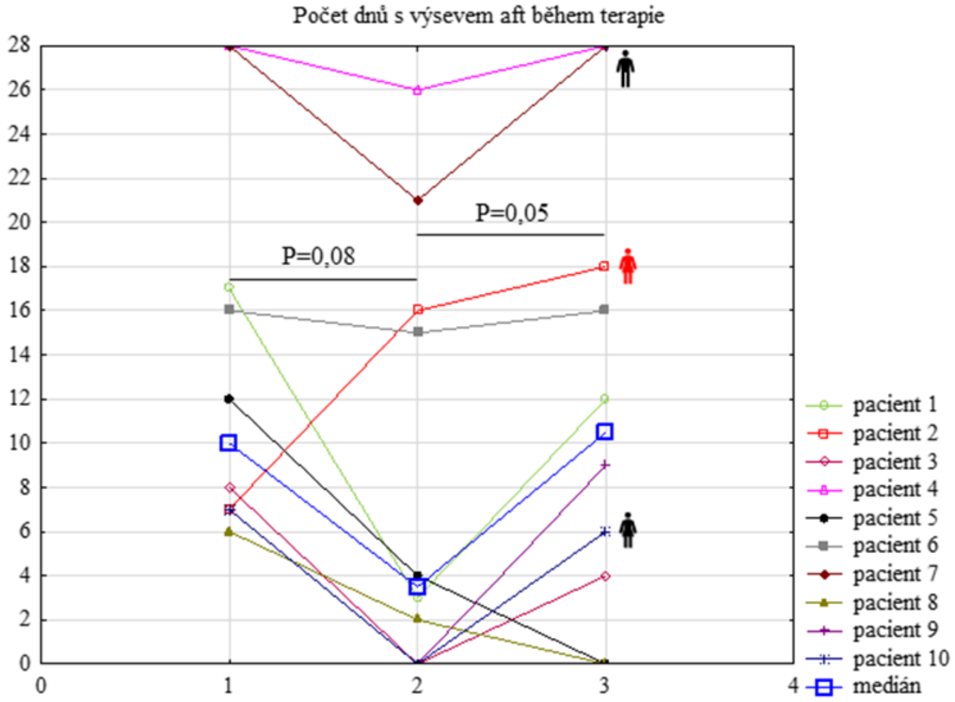
4=stav po 2. fázi

5=stav po 3. (sledovací) fázi

Z laboratorní analýzy vyplynulo, že hodnoty krevního obrazu, koncentrace homocysteinu, kyseliny listové, ALT, AST i dalších parametrů byly u vyšetřovaných osob ve fyziologickém rozmezí před terapií i po ní. U jedné pacientky byl před zahájením terapie zaznamenán zvýšený počet trombocytů, a to i při opakovaném vyšetření (až 506 $10^9\,l^{-1}$, kdy norma je 150–400 $10^9\,l^{-1}$), a proto byla žena odeslána na specializované hematologické oddělení.

Celkem 90 % pacientů při denních záznamech uvedlo, že v průběhu druhé (terapeutické) fáze měli nejméně dnů s výsevem aft (medián 3,5 dne [0–16 dnů]) oproti první (kontrolní) a třetí (sledovací) fázi (mediány deset dnů [7–17 dnů] a 10,5 dne [4–18 dnů], $p = 0{,}08$ a $p = 0{,}05$, hodnoceno Wilcoxonovým párovým testem), viz graf 2. Počet dnů s výsevem aft pozitivně koreloval mezi všemi třemi fázemi (korelační koeficenty = 0,792 a 0,765 a 0,905, $p \leq 0{,}01$,

hodnoceno Spearmanovým korelačním testem). Záznamy pacientů odpovídaly klinickému nálezu při vyšetřeních.



Graf 2. Počet dnů s výsevem aft během terapie

Hodnoceno Wilcoxonovým párovým testem.

černá/červená osoba= neutrální/negativní hodnocení terapie subjektem

Osa x

1=stav v průběhu 1. (kontrolní) fáze

2= stav v průběhu 2. (terapeutické) fáze

3=stav v průběhu 3. (sledovací) fáze

Podle subjektivního hodnocení pacientka č. 2 udala zhoršení stavu, pacienti č. 7 a 10 nepozorovali během terapie změnu svého zdravotního stavu a sedm osob bylo spokojeno, neboť u nich došlo k méně častému/žádnému výsevu aft a/nebo doba hojení byla významně kratší (graf 1). Subjektivní hodnocení terapie pozitivně korelovalo s objektivním klinickým nálezem v průběhu druhé (terapeutické) fáze (korelační koeficent = 0,993, $p < 0,001$, hodnoceno Spearmanovým korelačním testem). U pacientky č. 2, která bezprostředně po terapii uvedla nespokojenost s terapií, byl po dvou měsících od ukončení terapie klinický nález bez výsevu aft. Tato žena měla opakovaně nejnižší koncentrace vitaminu B_{12} z celé sledované skupiny (161–197 $pmoll^{-1}$; norma je 145–569 $pmoll^{-1}$). U pacienta č. 7, který je PM a nepozoroval během terapie změnu v souvislosti s RAS, bylo uvedeno, že byl celé tři měsíce permanentně ve stresu a téměř permanentně (77 dní z 84) měl difuzní výsev aft. Tento muž uvedl, že více než každý druhý den požíval alkohol. Pacientka č. 10 měla při všech měřeních sérové koncentrace 25-OH D_3 pod dolní hranicí normy, pouze v případě odběru měsíc po podání suplementu byla stanovena hodnota 50,2 $nmoll^{-1}$.

DISKUSE

Vitamin D a folát regulují řadu mechanismů, které slouží jako ochrana organismu před potenciálními environmentálními stresory. Nedávné poznatky shrnuté v práci od Jones a kol. [8] ukazují na interakce mezi UV zářením, typem kůže, vitaminem D_3, foláty a variabilitou v genech kódujících receptor pro vitamin D a enzymy zapojené do folátového cyklu. Existuje hypotéza nazvaná „vitamin D-folát“, podle které se zbarvení kůže vyvinulo jako vyrovnávací mechanismus pro udržení koncentrací obou vitaminů [8, 9]. UV záření na jedné straně stimuluje produkci vitaminu D v kůži, ale také může způsobit degradaci folátu při jeho absorpci a/nebo oxidaci volnými radikály.

Současná doporučení pro podávání vitaminu D a kyseliny listové jsou využívána především v prevenci řady onemocnění, neboť jejich deficit je spojován s rizikem a nástupem mnoha chronických stavů, jakými jsou kardiovaskulární nemoci, diabetes mellitus a nádorová onemocnění. Koncentrace zejména vitaminu D je odlišná v různých ročních obdobích, a po zimě je zpravidla nejnižší. Rozhodli jsme se proto, že námi navrženou terapii pacienti s RAS podstoupí jednotně na jaře.

S ohledem na genetickou predispozici ke snížené funkci enzymu MTHFR u osob zařazených do studie jsme pacientům podávali aktivní folát, konkrétně glukosaminovou sůl 5-methylfolátu, která je oproti předchozí generaci folátů (vápenné soli 5-methylfolátu) dlouhodobě stabilní, má vysokou rozpustnost ve vodě, lepší biologickou dostupnost a bezpečnost. Nepřipravili jsme glukosaminovou sůl 5-methylfolátu a vitaminem B~~6~~ spolu s vitaminem D_3 do jedné lékové formy nejenom kvůli jejich odlišným chemickým vlastnostem (stabilita by nebyla optimální), ale i proto, že jsme nejprve museli laboratorně ověřit (koncentrace Ca^{2+} a funkce ledvin), že pacientům bude možné vitamin D_3 podat. Lactosum monohydricum jsme zvolili kvůli jeho vlastnostem a také proto, že je jako pomocná látka obsažena v podávané tabletě s vitaminem D_3. Vitamin B_{12} jsme pacientům neaplikovali jednak z důvodů nutnosti podávání obden (intramuskulárně 300 µg), ale také proto, že koncentrace tohoto vitaminu byla před terapií u všech osob v normě (i po terapii byly hodnoty u všech pacientů ve fyziologickém rozmezí).

První (kontrolní) fázi jsme do terapie zařadili proto, abychom eliminovali tzv. carry-over effect, neboli přenos léčebného efektu léčby z předchozího období. Toto měsíční období by se dalo nazvat i „wash-out“ fází vyloučení vitaminových preparátů z organismu. Pacienti proto vysadili potravinové doplňky a neužívali jiné vitaminové preparáty než ty, které jsme jim při terapii podali.

Zvolený metodický přístup je vhodný pouze pro studium chronických, život neohrožujících onemocnění, jako je například RAS. Výhodou tohoto designu studie je, že lze porovnávat období bez užívání vitaminových preparátů s účinkem terapie u stejné osoby. Tento přístup vyžaduje menší počet respondentů než studie, při které jedna skupina obdrží placebo a druhá skupina léčivý preparát. Obvyklou nevýhodu tohoto přístupu, že lze studovat jen krátkodobé účinky léčby, jsme eliminovali zařazením sledovací fáze. Žádný z pacientů neodstoupil v

průběhu terapie ze studie, což může ukazovat na jejich důvěru v ošetřujícího zubního lékaře a u většiny i spokojenost s terapií a zlepšením stavu v průběhu druhé fáze.

Za jeden z důležitých faktorů v etiopatogenezi RAS je považována také psychika pacientů. Pozitivně mohla být ovlivněna už samotnou účastí ve studii a péčí, která jim byla věnována. Na druhou stranu je však možné i její negativní působení a vyvolání určité míry stresu, dané opakovanými návštěvami zubní ordinace s odběry žilní krve i nutností podrobně vyplňovat záznamové archy. Expozici stresu v průběhu terapie pacienti subjektivně hodnotili a zaznamenávali; výsledky jsou velmi variabilní (0–28 dní v každé z fází, minimum–maximum).

U 80 % pacientů s RAS byla sérová koncentrace 25-OH D_3 při ukončení terapie v normě (graf 1), což mohlo být důsledkem jednak ročního období s řadou slunných dnů (jaro/léto), ale také podání suplementu vitaminu D_3. Ve druhé (terapeutické) fázi byl v celé sledované kohortě nejnižší medián počtu dnů s výsevem aft (graf 2) a 70 % osob subjektivně pozorovalo zlepšení svého zdravotního stavu (tab. 1). Terapii RAS s použitím modifikované Škachovy kúry tak lze považovat za úspěšnou. Pouze u pacientky č. 2 byla terapie neúspěšná. Její stav se v průběhu léčebné fáze zhoršil (graf 2), a to jak subjektivně, tak i na základě objektivních vyšetření. Sérová koncentrace 25-OH D_3 se po podání tablety tohoto vitaminu v následujícím měsíci snížila a v dalším období skokově zvýšila nad normu (graf 1). Důvody nejsou jasné, jedním z možných vysvětlení může být i chyba při laboratorní analýze. Nicméně tato pacientka je nyní (dva měsíce) po ukončení studie objektivně bez nového výsevu aft. U pacienta č. 7, který je PM, se stav nezměnil. Ačkoliv došlo k mírnému zlepšení v průběhu druhé (terapeutické) fáze, forma a frekvence výsevu aft je u něj stále závažná, což může být i důsledkem jeho životního stylu (permanentní stres, alkohol). Pacientka č. 10, která terapii hodnotila neutrálně, neměla výsev aft ve druhé (terapeutické) fázi, ale subjektivně nepozorovala zlepšení. Její sérové koncentrace 25-OH D_3 jsou i po suplementaci na spodní hranici normy.

Všem pacientům zařazeným do studie jsme doporučili, aby vzhledem ke své genetické predispozici ke snížené aktivitě enzymu MTHFR v užívání aktivního folátu a vitaminu B_6 pokračovali, a budeme je dále sledovat. Domníváme se, že je vhodné u nich kontrolovat také koncentraci vitaminu D_3 v séru a při jejím poklesu podat vitaminový suplement.

Pilotní projekt plánujeme rozšířit o další pacienty s RAS. Modifikovanou Škachovu vitaminovou kúru by bylo vhodné vyzkoušet v různých časových (případně dávkových) schématech. Vitamin D_3 by mohl být podáván i pacientům, kteří jsou normální metabolizátoři kyseliny listové (EM), při snížených sérových koncentracích 25-OH D_3 a za nepřítomnosti kontraindikací. Navržený terapeutický přístup není kauzální léčbou, může však být považován za personalizovanou profylaxi. Pro pochopení etiopatogeneze onemocnění RAS a následnou optimalizaci léčby je nutný další výzkum.

ZÁVĚR

Navržená modifikace Škachovy vitaminové kúry se zdá být vhodná pro léčbu pacientů s RAS, u kterých je genetická predispozice ke snížené funkci enzymu MTHFR, neboť během našeho pozorování došlo u 70 % pacientů k subjektivnímu i objektivnímu zlepšení stavu. Doporučujeme zvážit provedení analýzy variability v genu MTHFR se snahou docílit individualizace léčebného postupu zejména u pacientů s RAS, kteří na standardně podávanou terapii neodpovídají.

LITERATURA

1. **Slezák R, Kopecký O, Krejsek.** Recidivující afty. Praha, Galén, 2000.

2. **Bořilová Linhartová P, Valová S, Izakovičová Hollá L.** Vrozená náchylnost k recidivující aftózní stomatitidě. Čes Stomat. 2017; 117(2): 27–34. ISSN 1210-7891.

3. **Škach M, Švejda J, Liška K.** Onemocnění ústní sliznice: patologie, terapie a diferenční diagnostika onemocnění ústní sliznice. Praha, Avicenum, 1975.

4. **van der Put NM, Gabreëls F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, van den Heuvel LP, Blom HJ.** A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet. 1998; 62(5): 1044–1051.

5. **Weisberg I, Tran P, Christensen B, Sibani S, Rozen R.** A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab. 1998; 64(3): 169–172.

6. **Ślebioda Z, Szponar E, Dorocka-Bobkowska B.** Vitamin D and its relevance in the etiopathogenesis of oral cavity diseases. Arch Immunol Ther Exp (Warsz). 2016; 64(5): 385–397.

7. **Anand A, Singh S, Sonkar AA, Husain N, Singh KR, Singh S, Kushwaha JK.** Expression of vitamin D receptor and vitamin D status in patients with oral neoplasms and effect of vitamin D supplementation on quality of life in advanced cancer treatment. Contemp Oncol (Pozn). 2017; 21(2): 145–151.

8. **Jones P, Lucock M, Veysey M, Beckett E.** The vitamin d-folate hypothesis as an evolutionary model for skin pigmentation: an update and integration of current ideas. Nutrients. 2018; 10(5): pii: E554. doi: 10.3390/nu10050554.

9. **Branda RF, Eaton JW.** Skin color and nutrient photolysis: an evolutionary hypothesis. Science. 1978; 201(4356): 625–626.

Článek vznikl s podporou Ministerstva zdravotnictví České republiky, grantu NV15-29336A, grantu GAČR GB14-37368G, projektu Specifický výzkum MUNI/A/1008/2017 a z prostředků poskytnutých Lékařskou fakultou MU juniorskému výzkumníkovi Petře Bořilové Linhartové.

Děkujeme studentům Natálii Mlčůchové, Mgr. Bc. Simoně Slezákové, Bc. Davidu Novákovi a zdravotním sestrám FN u sv. Anny, Brno za pomoc při odběru a zpracování vzorků.

RNDr. Petra Bořilová Linhartová, Ph.D.

Stomatologická klinika a Ústav patologické fyziologie

LF MU a FNUSA

Kamenice 5

625 00 Brno

e-mail: plinhart@med.muni.cz

Attachment 3 Article "Personalized therapy in patients with gastroesophageal reflux disease – metodology of *CYP2C19* gene profile´s determination"

doi: 10.14735/amgh2018320

Personalizovaná terapie pacientů s gastroezofageální refluxní chorobou – metodika stanovení genového profilu *CYP2C19*

Personalized therapy in patients with gastroesophageal reflux disease – methodology of *CYP2C19* gene profile's determination

P. Bořilová Linhartová[1,2], L. Bartošová[3], A. Křenek[4], L. Bartoš[5], J. Dolina[6], F. Marek[4], Z. Kala[4], L. Izakovičová Hollá[1,2]

[1] *Stomatologická klinika LF MU a FN u sv. Anny v Brně*
[2] *Ústav patologické fyziologie, LF MU, Brno*
[3] *Farmakologický ústav, LF MU, Brno*
[4] *Chirurgická klinika LF MU a FN Brno*
[5] *Ústav biochemie, PřF MU, Brno*
[6] *Interní gastroenterologická klinika LF MU a FN Brno*

Souhrn: Úvod: Konzervativní léčba refluxní choroby jícnu (GERD – gastroesophageal reflux disease) je v současnosti opřena o skupinu léků, které nejúčinněji potlačují sekreci kyseliny solné v žaludku, a to tzv. inhibitory protonové pumpy (IPP). I když se nejedná o kauzální léčbu onemocnění, jsou považovány a akceptovány jako léky volby „zlatého standardu". Cílem retrospektivní studie bylo zmapovat medikace pacientů s GERD v různých stupních onemocnění, analyzovat jejich individuální variabilitu v genu kódujícím enzym cytochrom P450 (CYP2C19) a navrhnout metodiku pro jednoduché stanovení genového profilu pacienta s GERD pro zefektivnění terapeutických postupů. **Metody:** Do studie bylo zařazeno celkem 276 osob s GERD po chirurgickém zákroku se známou farmakologickou anamnézou – 94 pacientů s neerozivní refluxní chorobou, 121 s refluxní ezofagitidou a 61 s Barrettovým jícnem (BE – Barrett's esophagus) nebo adenokarcinomem jícnu (EAC – esophageal adenocarcinoma). Stanovení genotypů dvou polymorfizmů genu *CYP2C19* (*17 rs12248560 a *2 rs4244285) bylo založeno na principu kvantitativní polymerázové řetězové reakce. **Výsledky:** Více než 90 % pacientů užívalo IPP (omeprazol/lansoprazol/pantoprazol). Ačkoli ve sledované kohortě předepsaná dávkování IPP odpovídala doporučení pro udržovací terapii, u pacientů nebyla zohledněna míra schopnosti účinnou látku metabolizovat. Bylo zjištěno, že nositelé genotypových kombinací obsahujících variantu *CYP2C9**17 determinující fenotyp ultrarychlého metabolizátoru (UM) mají nižší pravděpodobnost souběžného výskytu s variantou *CYP2C19**2, která kóduje fenotyp intermediárního nebo „špatného" metabolizátoru (IM nebo PM), než osoby se standardní funkcí enzymu CYP2C19 (p = 0,001). Frekvence kombinací genotypů (tzv. haplogenotypů) a ve výsledku fenotypů UM/IM/PM byla mezi pacienty s GERD 37,3/16,7/1,4 %. **Závěr:** U pacientů s GERD by měl být před zahájením farmakoterapie IPP definován jejich genový profil, resp. kombinace variant *CYP2C19**17 a *2, a to vzhledem k jejich vysoké frekvenci v populaci a funkčnímu metabolickému významu. Předpokládáme, že by toto opatření mohlo vést ke zvýšení efektivity farmakoterapie, a tím ke zlepšení kvality života pacientů a snad i k prevenci rozvoje závažnějších stavů, jakými jsou BE a EAC. Nestandardní schopnost metabolizovat IPP by mohla být jedním z předpokladů k indikaci chirurgického zákroku u pacientů s GERD.

Klíčová slova: farmakogenetika – gastroezofageální reflux – genový polymorfizmus – inhibitory protonové pumpy – pomalý metabolizátor – ultrarychlý metabolizátor – *CYP2C19**2 – *CYP2C19**17

Summary: Introduction: Conservative treatment of gastroesophageal reflux disease (GERD) is currently based on a group of drugs that effectively suppress the secretion of hydrochloric acid in the stomach, so-called proton pump inhibitors (PPI). Although these drugs do not target the cause of the disease, they are considered and accepted as the "gold standard" for the treatment of this disease. The aim of this retrospective study was to map medication of GERD patients in various phases of the disease, to analyze individual variability in the cytochrome P450 (*CYP2C19*) gene, and to propose an effective method for the simple determination of the gene in GERD patients. **Methods:** The study included 276 GERD patients with known pharmacological anamnesis who had undergone surgical treatment. The subjects included 94 patients with non-erosive reflux disease, 121 with reflux esophagitis, and 61 with Barrett's esophagus (BE) or esophageal adenocarcinoma (EAC). Genotypes of two polymorphisms in the *CYP2C19* gene (*17 rs12248560 and *2 rs4244285) were determined using quantitative polymerase chain reaction. **Results:** More than

90% patients were treated with PPI (omeprazole/lansoprazole/pantoprazole). Although the prescribed PPI dosing in the studied cohort was consistent with recommendations for maintenance therapy, the patients' ability to metabolize the active substance was not considered. Carriers of genotype combinations containing the *CYP2C9**17 variant, which determines the ultra-rapid metabolizer (UM) phenotype, were less likely to co-occur with the *CYP2C19**2 variant, which determines the intermediate or "poor" metabolizer (IM or PM) phenotype, than individuals with the standard functioning CYP2C19 enzyme (p = 0.001). The frequencies of combinations of genotypes (haplogenotypes) and resulting UM/IM/PM phenotypes were 37.3/16.7/1.4% in GERD patients. **Conclusion:** Prior to initiating PPI pharmacotherapy in GERD patients, determination of *CYP2C19* haplogenotypes (*CYP2C19**17 and *2 variants) should be performed due to frequent occurence of these variants in population and their functional metabolic significance. We presume that this approach will increase the effectiveness of pharmacotherapy, improve patient quality of life, and very likely help prevent/reduce the risk of the development of more serious conditions, such as BE and EAC. The non-standard ability to metabolize PPI in GERD patients may be one of the indicators for surgical intervention.

Key words: pharmacogenetics – gastroesophageal reflux – gene polymorphism – proton pump inhibitors – poor metabolizer – ultra rapid metabolizer – *CYP2C19**2 – *CYP2C19**17

Úvod

Gastroezofageální refluxní choroba (GERD – gastroesophageal reflux disease) je chronické onemocnění, které v západních zemích postihuje 10–20 % dospělé populace. Endoskopicky negativní/neerozivní refluxní nemoc jícnu (NERD – nonerosive reflux disease) je přítomna u významné části (až 50 %) pacientů s refluxními potížemi. Symptomy mohou být obvykle dobře kontrolovatelné terapií, dlouhotrvající průběh choroby významně zvyšuje riziko rozvoje zánětu refluxní ezofagitidy (RE), intestinálních metaplastických změn Barrettova jícnu (BE) a adenokarcinomu ezofagu (EAC – esophageal adenocarcinoma).

Léčba onemocnění je založena na dietních opatřeních, farmakoterapii a případně endoskopické léčbě a chirurgickém zákroku, který je indikován velmi často jako alternativa dlouhodobé medikamentózní terapie či při významné regurgitaci s rozvojem extraezofageálních příznaků.

Farmakoterapie u pacientů s GERD

Při farmakologické terapii jsou pacientům s GERD předepisována léčiva ze skupiny inhibitorů protonové pumpy (IPP), antacid, alginátů, prokinetik a antagonistů histaminových receptorů 2 (H2RA), příp. jejich kombinace. V současnosti nejúčinnější a nejvíce využívané v léčbě jsou IPP, které ireverzibilně blokují H^+/K^+ ATPázu protonové pumpy v parietálních buňkách žaludeční sliznice, což ovlivňuje sekreci vodíkových iontů, a tedy i pH.

Zatímco u pacientů s GERD bez RE jsou výsledky dlouhodobé léčby IPP a podávání IPP dle aktuální potřeby (tzv. terapie na vyžádání – on demand) srovnatelné, tak při léčbě GERD se souběžnou RE je kontinuální léčba IPP efektivnější [1]. Na druhou stranu se při dlouhodobém užívání, které je obvyklé vzhledem k chronické povaze onemocnění, mohou objevit nežádoucí účinky terapie, jako je osteoporóza v důsledku sníženého vstřebávání minerálů, snížené vstřebávání vitaminu B_{12} a železa, a dále pak, zejména u starších pacientů, např. chronické onemocnění ledvin nebo demence [2]. U nově vyvíjených molekul ze skupiny IPP je proto snaha zlepšit jejich farmakodynamické a farmakokinetické vlastnosti, např. časově rozvolnit efekt léčiva na změnu pH, a tím snížit výskyt nežádoucích účinků z toho pramenících; případně snížit jejich interakční potenciál daný účastí cytochromálních enzymů v metabolizmu [3].

Vzhledem k zásadnímu významu IPP ve farmakoterapii pacientů s GERD hraje velký význam identifikace faktorů, které mohou neúspěšnost této léčby předem predikovat. Mezi tyto faktory se řadí funkční poruchy trávení u pacientů s GERD [4], NERD [5] nebo nestandardní metabolizace IPP. Neopomenutelným faktorem zůstává také nedostatečná adherence pacientů k léčbě [6]. Klíčovým nástrojem pro potvrzení podezření, že pacient patří mezi nonrespondéry na IPP terapii, je u pacientů s GERD 24hodinová pH-metrie jícnu (monitoring pH-impedance) [7,8].

Metabolizace IPP a lékové interakce

Všechny IPP (omeprazol, lansoprazol, pantoprazol, esomeprazol, rabeprazol a dexlansoprazol) jsou metabolizovány v játrech enzymy cytochromu P450, jejichž aktivita je ovlivněna jak vnějšími, tak i vnitřními faktory. Na biotransformaci se primárně podílí izoenzym CYP2C19, v menší míře také CYP3A4, přičemž význam izoenzymu CYP3A4 narůstá právě u jedinců s geneticky nefunkčním CYP2C19 [9].

CYP2C19 je přitom zodpovědný nejen za metabolizaci IPP, ale i řady dalších běžně užívaných léčiv ze skupiny antiepileptik, antidepresiv, antimykotik a antiagregancií (např. klopidogrel). Interakční potenciál s klopidogrelem je nejvyšší u omeprazolu, zatímco u rabeprazolu je nejnižší ze všech IPP [10]. Vzhledem k tomu, že IPP zvyšují intragastrické pH, mohou ovlivnit množství neionizované frakce, a tedy vstřebávání některých léčiv. U slabých kyselin je rozpustnost a vstřebávání zvýšena, naopak u slabých bazí snížena. Většina dosud popsaných lékových interakcí IPP se však vysvětluje na úrovni ovlivnění jejich metabolizmu, jedná se o inhibici nebo o indukci izoenzymů CYP2C19 nebo CY3A4. Některé z IPP jsou navíc samy také inhibitory CYP2C19, např. omeprazol [11].

Genové varianty *CYP2C19* a jejich klinický dopad

Změna aktivity enzymu CYP2C19 nemusí být způsobena pouze shora uvedenými lékovými interakcemi, ale i genetickou dispozicí jedince [12,13]. V odborné literatuře je pozornost věnována především asociaci mezi genotypem *CYP2C19* pacienta a mírou eradikace *Helicobacter pylori* [14].

Do současnosti bylo v genu kódujícím enzym CYP2C19 nalezeno > 49 variantních alel, klinický dopad je však znám pouze u 9 z nich. Jen u jedné varianty (označované jako *17 rs12248560) byl zjištěn ultrarychlý metabolizmus IPP v důsledku bodové mutace v regulační části genu a následně zvýšené genové expresi a aktivitě enzymu. Alela *CYP2C19**17 predikuje nositele k neefektivnímu účinku standardních dávek IPP; fenotypově se jedná o tzv. ultrarychlé metabolizátory (UM). Všechny ostatní variantní alely jsou charakterizovány sníženou aktivitou enzymu CYP2C19 nebo úplnou ztrátou jeho enzymatické funkce (*2, *2B, *3, *4, *6, *7, *8). Mezi těmito je populačně nejčetnější variantní alela *CYP2C19**2 (rs4244285). Tato bodová mutace, charakteru záměny guaninu za adenin v pozici 681 v exonu 5, je zodpovědná za vznik nového aberantního místa sestřihu v aminokyselinové sekvenci genu, což vede k expresi zkráceného a nefunkčního proteinu CYP2C19 [15]. Homozygotní nositelé dvou standardních alel jsou označováni jako extenzivní metabolizátoři (EM), osoby s jednou variantní alelou jako intermediární metabolizátoři (IM), což se klinicky projeví zpomalením metabolizmu substrátů tohoto enzymu. Pokud jsou přítomny dvě variantní alely *2*2, vede to ke kompletnímu deficitu enzymatické aktivity CYP2C19 a tito nositelé jsou označováni jako pomalí metabolizátoři (PM) [12], i když příznačnější by pravděpodobně bylo označení „špatní“ metabolizátoři.

O výsledném fenotypu, resp. funkční aktivitě enzymu CYP2C19, tak rozhoduje vzájemná kombinace přítomnosti variantních alel. Jelikož je exprese *CYP2C19* významně ovlivněna genovými polymorfizmy, je u léčiv jím metabolizovaných uvedeno upozornění na tuto skutečnost nebo preskripční omezení na stránkách Úřadu pro kontrolu potravin a léčiv (FDA – Food and Drug Administration) [16].

Cílem naší studie bylo:

1. zmapovat užívání léčiv u pacientů s různými stupni GERD,
2. analyzovat jejich individuální variabilitu v genu kódujícím CYP2C19,
3. navrhnout metodiku pro jednoduché stanovení genového profilu pacienta s GERD pro zefektivnění terapeutických postupů.

Soubor pacientů a metodika

Do retrospektivní studie s designem „case-case“ (porovnávání skupin pacientů NERD vs. RE/BE + EAC i RE vs. BE + EAC) bylo zahrnuto 276 osob (z toho 94 pacientů s NERD, 121 jedinců s RE a 61 pacientů s BE nebo EAC), které v letech 2004–2011 podstoupily zákrok (laparoskopickou fundoplikaci pro GERD a BE, resekční zákrok pro EAC) na Chirurgické klinice LF MU a FN Brno. Pacienti byli k zákroku odesíláni z Interní gastroenterologické kliniky LF MU a FN Brno; GERD jim byla diagnostikována na základě klinických symptomů, jako je pálení žáhy a/nebo regurgitace kyseliny s objektivizací pomocí 24hodinové pH-metrie, ezofagogastroduodenoskopie, manometrie jícnu a histologického vyšetření. Patologický kyselý reflux byl definován dle DeMeester score nad 14,7. Kritéria pro zařazení do studie byla následující: osoby české národnosti starší 18 let, diagnóza GERD s následnou chirurgickou terapií, dostupné údaje o farmakoterapii. Vyloučeni byli pacienti s achalázií nebo s divertikly jícnu.

V souladu s Helsinskou deklarací pacienti před zařazením do studie podepsali informovaný souhlas schválený Etickou komisí FN Brno (NPV 2B 06060).

Genetická analýza

Pacientům bylo odebráno 9 ml periferní krve do zkumavky s protisrážlivým činidlem (EDTA). Genomová DNA byla izolována z leukocytů pomocí fenol-chloroformové extrakce s použitím izopropanolu a proteinázy K na Ústavu patologické fyziologie, LF MU Brno. Na principu kvantitativní řetězové reakce v reálném čase byly stanoveny dva jednonukleotidové polymorfizmy (SNP – single nucleotide polymorphisms) v *CYP2C19* (*17 rs12248560 a *2 rs4244285) pomocí fluorescenčně značených sond 5′ nuclease TaqMan® assay (C_469857_10 a C_25986767_70, Thermo Fisher Scientific, Waltham, Massachusetts, USA) dle protokolu od výrobce. Fluorescence byla měřena pomocí přístroje LightCycler 96 (Roche Diagnostics, Basilej, Švýcarsko) a fluorescenční změny v reálném čase byly analyzovány pomocí softwaru LightCycler 96 Application Software a LightCycler 96 Instrument.

Statistická analýza

Významnost rozdílů v alelických frekvencích mezi jednotlivými skupinami byla hodnocena Fisherovým testem. Signifikance odchylek od Hardy-Weinbergovy rovnováhy pro každý polymorfizmus a rozdíly ve frekvencích genotypů (i jejich kombinací, tzv. haplogenotypů) jsme testovali pomocí χ^2-testu. Haplotypová analýza byla provedena pomocí programu SNP analyzer v. 2.0 (http://snp.istech21.com/snpanalyzer/2.0/). Rozdíly ve frekvencích haplotypů byly počítány permutačním testem a hodnota $p < 0,05$ byla považována za statisticky významnou.

Výsledky

Demografický popis studované populace vč. farmakoterapeutických údajů je uveden v tab. 1. Věkový průměr 276 pacientů s GERD zařazených do studie v době hospitalizace byl 46,4 ± 13,4 let, z toho bylo 166 mužů (ve věku 44,9 ± 13,3 let) a 110 žen (ve věku 48,6 ± 12,6 let). Průměrný body mass index (BMI) byl u pacientů s GERD 26,3 ± 3,3 kg/m^2. BE nebo EAC byli častěji postiženi muži (72,1 %). Před operací byl u 89,9 % pacientů s GERD přítomen ky-

Tab. 1. Demografický popis studované populace a farmakoterapie GERD.
Tab. 1. Demographic description of the studied population and pharmacotherapy of GERD.

	NERD	RE	BE + EAC
Počet pacientů (n)	94	121	61
Pohlaví – muži (%)	45 (47,9)	77 (63,6)	44 (72,1)
Věk – průměr ± SD (roky)	44,5 ± 14,1	45,0 ± 12,2	52,0 ± 12,1
***H. pylori* (%)**	6 (6,4)	14 (11,6)	3 (4,9)
IPP (%)	85 (90,4)	115 (95,0)	51 (83,6)
OME (%)	46 (48,9)	77 (63,6)	34 (55,7)
LANSO (%)	17 (18,1)	19 (15,7)	8 (13,1)
PANTO (%)	11 (11,7)	8 (6,6)	4 (6,6)
Prokinetika (%)	36 (38,3)	49 (40,5)	18 (29,5)
Polypragmázie (%)	17 (18,1)	15 (12,4)	13 (21,3)

GERD – gastroezofageální refluxní choroba, NERD – neerozivní refluxní choroba jícnu, RE – refluxní ezofagitida, BE + EAC – Barrettův jícen + adenokarcinom jícnu, *H. pylori* – *Helicobacter pylori*, IPP – inhibitor protonové pumpy, OME – omeprazol, LANSO – lansoprazol, PANTO – pantoprazol, SD – směrodatná odchylka, n – počet

Tab. 2. Genotypové frekvence *CYP2C19* u pacientů s GERD.
Tab. 2. *CYP2C19* genotype frequencies in patients with GERD.

	NERD	RE	BE + EAC
***CYP2C19*17* (rs12248560)**			
CC (EM) *1*1	50 (53,2)	66 (54,5)	36 (59,0)
CT (UM) *1*17	39 (41,5)	47 (38,8)	20 (32,7)
TT (UM)*17*17	5 (5,3)	8 (6,6)	5 (8,2)
MAF	49 (26,0)	63 (25,8)	30 (24,6)
***CYP2C19*2* (rs4244285)**			
GG (EM) *1*1	69 (73,4)	91 (75,2)	45 (73,8)
GA (IM) *1*2	24 (25,5)	29 (24,0)	14 (23,0)
AA (PM) *2*2	1 (1,1)	1 (0,8)	2 (3,3)
MAF	26 (13,8)	31 (12,7)	18 (14,8)
***CYP2C19*17/ CYP2C19*2* Haplogenotyp**			
TTGG (UM) *17*17/*1*1	5 (5,3)	8 (6,6)	5 (8,2)
CTGG (UM) *1*17/*1*1	29 (30,9)	39 (32,2)	17 (27,9)
CTGA (AM) *1*17/*1*2	10 (10,6)	8 (6,6)	3 (4,9)
CCGG (EM) *1*1/*1*1	35 (37,2)	44 (36,4)	23 (37,7)
CCGA (IM) *1*1/*1*2	14 (14,9)	21 (17,4)	11 (18,0)
CCAA (PM) *1*1/*2*2	1 (1,1)	1 (0,8)	2 (3,3)

GERD – gastroezofageální refluxní choroba, NERD – neerozivní refluxní choroba jícnu, RE – refluxní ezofagitida, BE + EAC – Barrettův jícen + adenokarcinom jícnu, CYP2C19 – cytochrom P450 2C19, PM – „špatný" metabolizátor, IM – intermediární metabolizátor, EM – extenzivní (normální) metabolizátor, AM – ambivalentní metabolizátor, UM – ultrarychlý metabolizátor, MAF – frekvence minoritní alely

selý reflux, zatímco po operaci byl prokázán pouze u 15,2 % osob ($p < 0,000001$). U 12 pacientů došlo k progresi onemocnění do závažnějšího stadia onemocnění a naopak u 7 k objektivnímu zlepšení v průběhu sledovaného období (v rozmezí let 2004–2011). U 3 pacientů se stav z původní diagnózy NERD rozvinul do EAC, ani jeden z těchto pacientů neužíval IPP.

Farmakoterapie

Z dostupných lékařských záznamů vyplynulo, že 45 pacientů s GERD (16,3 %) z našeho souboru užívá současně více než pět léčivých přípravků (tab. 2), což je definováno jako polypragmazie. Celkem 90,9 % pacientů sledované kohorty užívalo některé léčivo ze skupiny IPP, 103 pacientů (37,3 %) bralo prokinetika (itoprid, metoklopramid). Pouze jeden pacient užíval léčivo ze skupiny H2RA (ranitidin). Mezi dalšími byla nejčastěji zastoupena léčiva ovlivňující kardiovaskulární systém (antihypertenziva, antiarytmika a organické nitráty), která užívalo 23,6 % pacientů, a psychofarmaka (antidepresiva, antiepileptika a anxiolytika) užívaná 13,3 % pacientů s GERD. V námi sledované kohortě užívalo 19 pacientů (6,9 %) současně s IPP jiné léčivo (citalopram, escitalopram, sertralin, fluvastatin, fenofibrát, celekoxib a methotrexát), u něhož je popsáno potenciální riziko lékové interakce na úrovni metabolizmu.

Ze skupiny IPP byl nejčastěji (62,5 %) pacientům s GERD podáván omeprazol s mediánem denní dávky 20 mg (min.–max., 20–60 mg), dále pak v 17,5 % lansoprazol s mediánem denní dávky 30 mg (30–60 mg) nebo v 9,2 % pantoprazol s mediánem denní dávky 40 mg (20–40 mg). U dalších 13,1 % pacientů byl předepsán jeden z těchto IPP, v záznamech však chyběl údaj o konkrétním typu IPP a zvoleném dávkování. U 7 pacientů došlo v průběhu užívání ke změně typu IPP. Pozitivní nález *H. pylori* byl zaznamenán u 8,3 % pacientů a medián dávkování omeprazolu u nich byl 20 mg/den (20–60 mg), u 1 pacienta byl předepsán pantoprazol v dávkování 40 mg/den.

Genový profil *CYP2C19*

Nezaznamenali jsme rozdíly ve frekvencích alel ani genotypů u obou studovaných SNP mezi jednotlivými

skupinami pacientů s GERD, oba hodnocené SNP byly v Hardy-Weinbergově rovnováze (p > 0,05) (tab. 2). Ve studované populaci bylo zastoupeno 44,9 % pacientů, kteří nesou alespoň jednu alelu *CYP2C19**17 (rs12248560), a 25,7 % nositelů alespoň jedné alely *CYP2C19**2 (rs4244285). Na základě haplogenotypové analýzy byl zjištěn signifikantní rozdíl ve frekvencích variant kódujících EM a IM + PM vs. UM a ambivalentní metabolizátor (AM) (p = 0,001) (tab. 3); v naší práci jsme fenotyp u pacientů s haplogenotypem *1*17/*1*2 originálně označili zkratkou AM. Zastoupení pacientů s GERD a současně s fenotypem AM bylo 7,6 %. Analýza vazebné nerovnováhy ukázala, že byly varianty *CYP2C19**2 a *CYP2C19**17 v kompletní vazebné nerovnováze (|D'| = 1,0).

Tab. 3. Haplogenotypové frekvence *CYP2C19* u pacientů s GERD.
Tab. 3. *CYP2C19* haplogenotype frequencies in patients with GERD.

		*CYP2C19**2 (rs4244285)		
		*1*1	*1*2	*2*2
***CYP2C19**17 (rs12248560)**	***1*1**	102 (37,0)	46 (16,7)	4 (1,4)
	***1*17**	85 (30,8)	21 (7,6)	0
	***17*17**	18 (6,5)	0	0

GERD – gastroezofageální refluxní choroba, CYP2C19 – cytochrom P450 2C19

Užívání IPP ve vztahu k fenotypu CYP2C19

Více než 10 % pacientů s fenotypem UM (dle haplogenotypu), kterých bylo celkem 103, neužívalo žádný IPP. Ti, kteří v rámci farmakoterapie měli IPP předepsán, užívali ve 49,5 % omeprazol s mediánem denní dávky 20 mg (20–60 mg), případně lansoprazol/pantoprazol s mediánem 30 mg/den (30–60 mg), resp. 40 mg/den (20–40 mg). Osm pacientů s fenotypem UM a s pozitivním nálezem *H. pylori* mělo předepsáno dávkování omeprazolu s mediánem denní dávky 40 mg (20–40 mg) nebo 40 mg pantoprazolu za den. Všichni 4 pacienti s fenotypem PM (dle haplogenotypu) užívali IPP, a to omeprazol v denní dávce 40 mg. U 2 z těchto pacientů se rozvinul BE, 1 pacient měl RE a u 1 osoby byl zaznamenán NERD.

Diskuze

Až u 40 % pacientů s GERD přetrvávají příznaky refluxu, a to i přes terapii IPP. V recentní přehledové práci jsou shrnuty důkazy pro indikace chirurgického, resp. endoskopického přístupu k terapii GERD u pacientů nereagujících na konzervativní léčbu s podáváním IPP. Laparoskopická fundoplikace dle autorů poskytuje prokazatelnou úlevu od symptomů až na 10 let, i když její účinnost časem klesá [17]. Naše výsledky po měření pH-impedance u pacientů s GERD před operací a po ní jsou rovněž důkazem o přínosu tohoto terapeutického přístupu.

Chirurgický zákrok u pacientů s GERD je indikován především u mladších pacientů, a proto je v naší studii celkové zastoupení pacientů s polypragmazií relativně nízké. Nejvíce osob s polypragmazií jsme zaznamenali ve skupině pacientů s BE + EAC (u 21 % z nich), což může souviset s jejich vyšším průměrným věkem (tab. 1), neboť se zvyšujícím se věkem stoupá polymorbidita. V populaci seniorů se uvádí incidence polypragmazie v rozmezí 30–60 % [18]. Téměř 90 % pacientů, kteří podstoupili chirurgický zákrok z indikace GERD, současně užívalo některý z IPP 1. generace, tedy omeprazol, lansoprazol nebo pantoprazol. Medián denní dávky omeprazolu byl 20 mg, což je dle guidelines při udržovací léčbě dávka standardní. Na druhou stranu je však u pacientů, kteří mají endoskopické změny na sliznici jícnu, doporučována výrazně vyšší denní dávka, a to 60–80 mg omeprazolu nebo 60 mg lansoprazolu nebo 80 mg pantoprazolu [19]. U pacientů s BE + EAC užívajících lansoprazol bylo toto doporučení dodrženo, zatímco v případě omeprazolu a pantoprazolu byl medián preskripce denní dávky pouze 40 mg.

Ačkoli můžeme konstatovat, že předepsaná dávkování IPP následovala doporučení, u pacientů nebyla zohledněna individuální míra schopnosti účinnou látku metabolizovat. Vyšetření schopnosti metabolizace IPP není v ČR standardně prováděno, i když je známo, že nejen volba vhodného dávkování, ale také konkrétního léčiva ze skupiny IPP nabývá u pacientů s variantními alelami v genu pro CYP2C19 zásadního významu.

Mezi jednotlivými IPP existují značné rozdíly v biotransformaci vzhledem k rozdílné míře, jakou se na metabolizmu jednotlivých IPP podílejí oba izoenzymy (CYP2C19 a CYP3A4), příp. nakolik se uplatňují jiné, neenzymatické cesty metabolizace [20,21]. Jelikož je rabeprazol primárně metabolizován neenzymaticky a enzymů CYP využívá pouze omezeně [22], tak význam individuální variability v genu *CYP2C19* má u pacientů užívajících rabeprazol menší význam ve srovnání např. s omeprazolem. Domníváme se proto, že rabeprazol by byl nejvhodnějším kandidátem ze všech IPP v současnosti registrovaných a distribuovaných v ČR pro léčbu GERD u pacientů s variantními alelami v genu *CYP2C19*, obzvláště u pacientů s fenotypy PM a UM. Rabeprazol je navíc spojován i s nejvyšší efektivitou eradikace *Helicobacter pylori* [14].

Nelze však říci, že rabeprazol je nejlepší IPP. Z výsledků recentní studie porovnávající účinnost a snášenlivost jednotlivých IPP v dávkách doporučovaných dle FDA vyplynulo, že např. pro hojení slizničních erozí a úlevu od pálení žáhy je nejvhodnější esomeprazol v dávce 40 mg/den. Dále autoři zjistili, že dexlansoprazol, rabeprazol a omeprazol vykazovaly nižší účinnost a snášenlivost než esomeprazol, pantoprazol nebo lansoprazol [23].

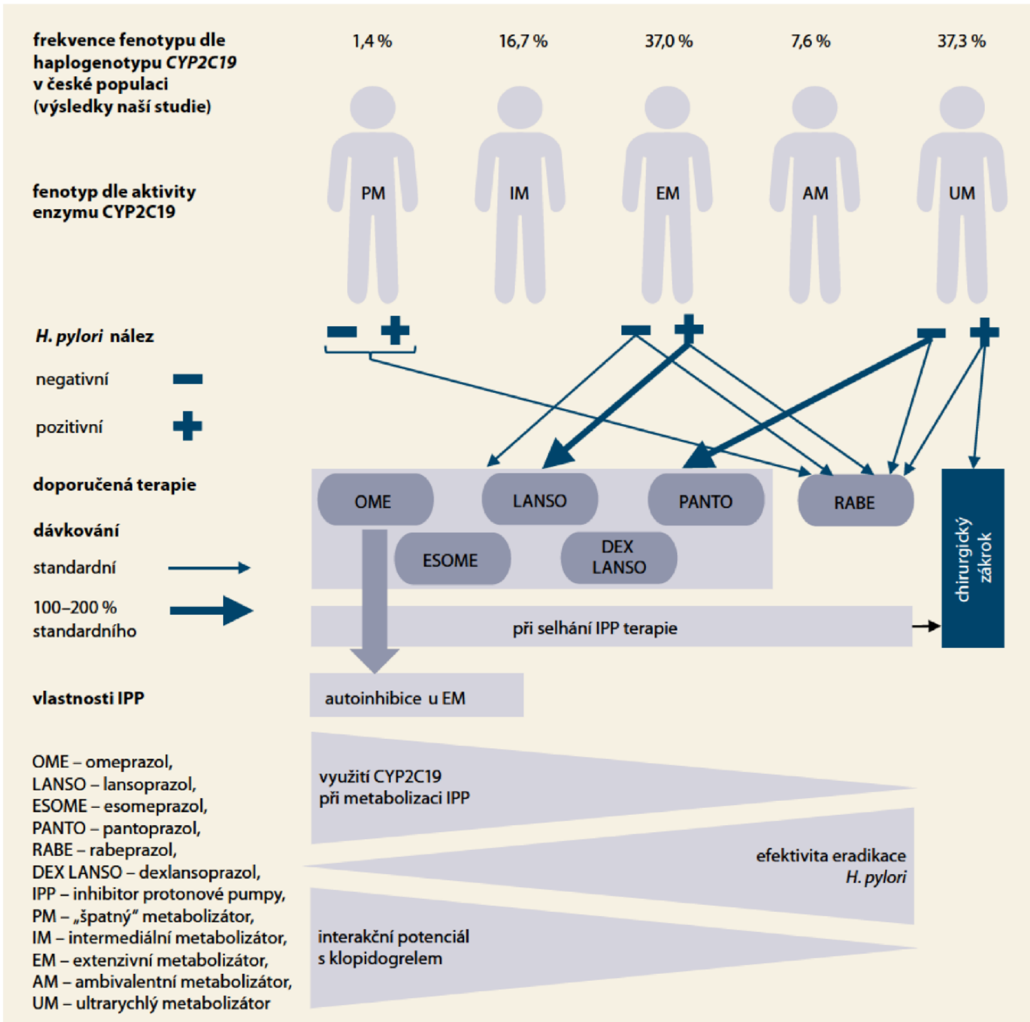


Schéma 1. Doporučená terapie u pacientů s GERD na základě stanoveného fenotypu CYP2C19.
Scheme 1. Recommended therapy in patients with GERD according to the CYP2C19 phenotype.

Je také známo, že při opakovaném podání omeprazolu se rozdíl ve farmakokinetice mezi pacientem s fenotypem EM a PM snižuje. Zatímco u EM dochází k inhibici CYP2C19 samotným omeprazolem (tzv. autoinhibice) a následně k částečné kumulaci léčiva, u PM k autoinhibici nedochází v důsledku nefunkčnosti enzymu CYP2C19 a částečná kumulace léčiva je dána jeho zpomaleným odbouráváním. Autoinhibice se neuplatňuje u lansoprazolu a pantoprazolu [11].

Dle doporučení FDA je u pacientů s fenotypem UM nebo při eradikaci *Helicobacter pylori* vhodné zvýšit denní dávku IPP na 100–200 % a pečlivě sledovat, zda je odpověď na terapii dostatečná. Pro ostatní fenotypy žádná doporučení k terapii IPP ze strany FDA nejsou [16,24]. Vzhledem ke skutečnosti, že zvolené dávkování omeprazolu i pantoprazolu bylo v našem souboru standardní, se lze domnívat, že u pacientů s GERD a fenotypem UM mohla být v důsledku jejich genetické dispozice zvolená léčba neúčinná/méně účinná. Rovněž u pacientů užívajících léčiva s interakčním potenciálem s IPP nebyl pravděpodobně výsledek terapie optimální.

IPP jsou nedílnou součástí jakéhokoli eradikačního schématu *Helicobacter py-*

lori, a to v dávkách 2× vyšších, než jsou standardní udržovací dávky. Metaanalýza studií zaměřených na účinnost eradikace v souvislosti s genetickými variantami *CYP2C19* zjistila významné rozdíly u jednotlivých skupin metabolizátorů užívajících omeprazol a lansoprazol, naopak u esomeprazolu a rabeprazolu (IPP 2. generace) nebyla účinnost eradikace ovlivněna. Výsledky klinického hodnocení eradikačních režimů dokumentují vyšší rychlost a vyšší úspěšnost eradikace u pacientů s fenotypem PM ve srovnání s EM [25,26].

V naší kohortě mělo pouze 23 pacientů pozitivní nález *Helicobacter pylori* a většina měla předepsáno dávkování IPP poloviční, než je doporučováno [19]. Jelikož 8 z nich patří navíc mezi UM, je zřejmé, že nastavená denní doporučená dávka IPP pro ně nebyla dostatečná. Právě genotypizace *CYP2C19* se tak může stát užitečným nástrojem pro rozhodování o optimálním eradikačním režimu *Helicobacter pylori* založeném na terapii IPP v kombinaci s antibiotickými preparáty.

U UM pacientů lze předpokládat, že mohou být nonrespondéry IPP terapie, proto by stanovení fenotypu mohlo být využito jako jeden z předpokladů k indikaci chirurgické léčby – zejména v případě, že jsou pacienti s GERD i *Helicobacter pylori* pozitivní. Naše výsledky, které jsou podloženy i závěry zahraničních studií zabývajících se IPP terapií u pacientů s GERD a možným efektem jejich individuální variability v genu *CYP2C19*, jsme shrnuli v doporučeních uvedených na schématu 1.

Legenda ke schématu 1

Po analýze genu CYP2C19 jsme zjistili, že v české populaci je 1,4 % pacientů, jejichž schopnost metabolizovat některé z IPP (omeprazol, esomeprazol, pantoprazol, dexlansoprazol, lansoprazol), je špatná z důvodu nízké aktivity jejich enzymu CYP2C19. Jedná se o PM, u kterých se farmakoterapie zahrnující rabeprazol jeví dle aktuálních poznatků jako nejlepší volba. Jelikož je toto IPP metabolizováno s minoritním využitím enzymu CYP2C19, je možné mít dávkování IPP více pod kontrolou, tím pádem i snížit nebezpečí nežádoucích účinků z důvodu předávkování, a rovněž lékových interakcí na úrovni cytochromu P450. Navíc u PM s GERD a pozitivním nálezem H. pylori je rabeprazol vhodný také s ohledem na jeho vysokou efektivitu při eradikaci H. pylori.

Více než 1/3 sledovaných pacientů s GERD má fenotyp EM, a tedy standardní aktivitu enzymu CYP2C19. Pro farmakoterapii těchto osob lze při negativním nálezu H. pylori předepsat jakékoli léčivo ze skupiny IPP. U EM (na rozdíl od PM) dochází k autoinhibici enzymu CYP2C19 při užívání omeprazolu, a proto je omeprazol pro EM vhodným léčivem s přihlédnutím na minimalizaci nežádoucích účinků. Pokud mají EM s GERD pozitivní nález H. pylori, pak je doporučováno zvýšit dávkování vybraných IPP (omeprazol, esomeprazol, pantoprazol, dexlansoprazol, lansoprazol) až na 200 % standardního dávkování dle SPC nebo opět zvolit rabeprazol ve standardním režimu dávkování.

Až 37,3 % pacientů s GERD, kteří fenotypově odpovídají UM, je v případě negativního nálezu H. pylori možno zaléčit všemi typy IPP, mělo by se však zvolit 100–200 % standardního dávkování dle SPC (s výjimkou rabeprazolu, kde dávkování může zůstat obvyklé). Ačkoli je u UM s pozitivním nálezem Helicobacter pylori možné předepsat rabeprazol, tak je vysoká pravděpodobnost neúspěchu farmakoterapie IPP, a proto navrhujeme indikaci chirurgického zákroku u této skupiny pacientů (při selhání terapie IPP a přetrvávajících obtížích lékaři přistupují u pacientů s GERD mimo jiné i k chirurgickému zákroku.)

U pacientů s IM, a především s AM fenotypem není aktivita enzymu CYP2C19 jednoznačně definovaná, a tudíž ani neexistují žádná klinická doporučení. Přikláníme se k tomu, že by u nich způsob terapie mohl kopírovat doporučení pro extrémní fenotypy, tedy PM, resp. UM.

Neočekávali jsme, že by mezi jednotlivými skupinami pacientů s GERD bylo významně vyšší/nižší zastoupení alel *CYP2C19**17 nebo *2, neboť sledované genové polymorfizmy nezasahují do patogeneze onemocnění. V evropské populaci je frekvence výskytu minoritní alely (MAF) *CYP2C19**2 (rs4244285) 14,5 % [27], v české populaci je to 13,0 % [28]. Data týkající se MAF *CYP2C19**17 v české populaci nejsou dosud k dispozici, pro evropskou populaci se uvádí hodnota 22,4 % [29]. Naše výsledky, kdy MAF pro alelu *2 byla 13,6 % a pro alelu *17 byla 25,7 %, odpovídají frekvencím v evropské populaci. Ukázalo se, že u pacientů po chirurgickém zákroku pro GERD je frekvence osob s abnormální metabolizací IPP shodná s frekvencí těchto fenotypů v celé populaci. Ačkoli by se nabízelo, že fenotyp abnormálního IPP metabolizátora bude ve skupině pacientů s GERD po laparoskopické fundoplikaci zastoupen častěji než u pacientů s GERD léčených jen konzervativně, tak naše výsledky nic takového nepotvrdily. Vysvětlení spočívá v tom, že k selhání IPP terapie dochází z vícero důvodů a že ne u všech osob, u kterých konzervativní léčba selhává, je vhodné indikovat chirurgický zákrok.

Podle práce Lewis et al [30] se variantní alela *CYP2C19**17 nevyskytuje v haplotypu společně s variantní alelou *CYP2C19**2, obě alelové varianty jsou v tzv. vazebné nerovnováze. Výsledky naší haplotypové/haplogenotypové analýzy tyto výsledky potvrzují. Recentní studie zaměřená na děti s GERD prokázala klinický význam varianty *CYP2C19**17 a její autoři ve shodě s námi doporučují před zahájením léčby IPP genetické testování [31], neboť zvýšená aktivita CYP2C19 (nositelé variantní alely *17), a tudíž zrychlené odbourávání účinné látky, může být příčinou selhání terapie. Pro jednoznačné definování fenotypu dle našeho názoru není dostačující stanovení pouze této jedné z variantních alel. Ačkoli mají léčiva ze skupiny IPP obecně dobrou toleranci [32] a geneticky podmíněné zvýšení koncent-

race u pacientů s fenotypem PM [33] se nemusí projevit toxicky, nelze zanedbat riziko nežádoucích účinků dlouhodobé terapie, které by mohlo být logicky u pacientů s tímto fenotypem vyšší. Z těchto důvodů doporučujeme stanovení polymorfizmů *CYP2C19*17* i *CYP2C19*2*, neboť zvýšení ekonomických nákladů je již minimální v poměru k benefitu, který tím pacient získá.

Dalším zajímavým aspektem stanovení genového profilu *CYP2C19* je, že při společné přítomnosti obou variantních alel s protichůdným vlivem na enzymatickou aktivitu CYP2C19 (haplogenotyp *1*17/*1*2) nemusí být fenotyp jednoznačný, označili jsme ho proto jako AM. Jelikož dosud nebyla u těchto AM jedinců definována aktivita enzymu CYP2C19, a tedy i jejich schopnost metabolizace IPP, je nutno efekt léčby IPP u těchto pacientů dále podrobněji analyzovat.

Závěr

Ačkoli jsou IPP velmi účinné a bezpečné léky s minimálním interakčním potenciálem, je nutno mít na paměti existenci genových polymorfizmů izoenzymu CYP2C19, které mohou být příčinou individuální variability farmakokinetických parametrů a vést k očividným rozdílům mezi pacienty z hlediska potlačení acidity, potenciálu pro vznik lékových interakcí a klinické účinnosti léčiva. Pochopení farmakokinetických rozdílů a přezkoumání možných alternativních cest metabolizmu může pomoci lékařům lépe individualizovat a optimalizovat léčbu IPP u nonrespondérů s GERD nebo u pacientů s vředovou chorobou po selhání eradikační terapie.

Jedná se o první studii zabývající se specifickou kombinací variant *CYP2C19**17 (rs12248560) a *CYP2C19**2 (rs4244285) v souvislosti s GERD a terapií IPP. Navrhujeme, aby tato jednoduchá a efektivní metodika stanovení genového profilu, která je založená na detekci dvou nejvíce frekventních funkčních variant genu kódujícího enzym CYP2C19, byla zvažována při léčbě IPP u pacientů s GERD.

Literatura

1. The Swedish Council on Technology Assessment in Health Care. Dyspepsia and gastro-oesophageal reflux. A systematic review. [online]. Available from: https://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0096647/pdf/PubMedHealth_PMH0096647.pdf.
2. Maes ML, Fixen DR, Linnebur SA. Adverse effects of proton-pump inhibitor use in older adults: a review of the evidence. Ther Adv Drug Saf 2017; 8(9): 273–297. doi: 10.1177/2042098617715381.
3. Mermelstein J, Mermelstein AC, Chait MM. Proton pump inhibitors for the treatment of patients with erosive esophagitis and gastroesophageal reflux disease: current evidence and safety of dexlansoprazole. Clin Exp Gastroenterol 2016; 9: 163–172. doi: 10.2147/CEG.S91602.
4. Garros A, Mion F, Marjoux S et al. Factors associated with nonresponse to proton pump inhibitors therapy in patients referred for esophageal pH-impedance monitoring. Dis Esophagus 2016; 29(7): 787–793. doi: 10.1111/dote.12374.
5. Bytzer P, van Zanten SV, Mattsson H et al. Partial symptom-response to proton pump inhibitors in patients with non-erosive reflux disease or reflux oesophagitis – a post hoc analysis of 5796 patients. Aliment Pharmacol Ther 2012; 36(7): 635–643. doi: 10.1111/apt.12007.
6. Domingues G, Moraes-Filho JP. Noncompliance is an impact factor in the treatment of gastroesophageal reflux disease. Expert Rev Gastroenterol Hepatol 2014; 8(7): 761–765. doi: 10.1586/17474124.2014.911660.
7. Kawamura O, Hosaka H, Shimoyama Y et al. Evaluation of proton pump inhibitor-resistant nonerosive reflux disease by esophageal manometry and 24-hour esophageal impedance and pH monitoring. Digestion 2015; 91(1): 19–25. doi: 10.1159/000368766.
8. Yadlapati R, Tye M, Roman S et al. Postprandial high-resolution impedance manometry identifies mechanisms of nonresponse to proton pump inhibitors. Clin Gastroenterol Hepatol 2018; 16(2): 211–218. doi: 10.1016/j.cgh.2017.09.011.
9. Hagymási K, Müllner K, Herszényi L et al. Update on the pharmacogenomics of proton pump inhibitors. Pharmacogenomics 2011; 12(6): 873–888. doi: 10.2217/pgs.11.4.
10. Scott SA, Owusu Obeng A, Hulot JS. Antiplatelet drug interactions with proton pump inhibitors. Expert Opin Drug Metab Toxicol 2014; 10(2): 175–189. doi: 10.1517/17425255.2014.856883.
11. Ogilvie BW, Yerino P, Kazmi F et al. The proton pump inhibitor, omeprazole, but not lansoprazole or pantoprazole, is a metabolism – dependent inhibitor of CYP2C19: implications for coadministration with clopidogrel. Drug Metab Dispos 2011; 39(11): 2020–2033. doi: 10.1124/dmd.111.041293.
12. Furuta T, Shirai N, Sugimoto M et al. Pharmacogenomics of proton pump inhibitors. Pharmacogenomics 2004; 5(2): 181–202. doi: 10.1517/phgs.5.2.181.27483.
13. Klotz U. Clinical impact of CYP2C19 polymorphism on the action of proton pump inhibitors: a review of a special problem. Int J Clin Pharmacol Ther 2006; 44(7): 297–302.
14. Lin YA, Wang H, Gu ZJ et al. Effect of CYP2C19 gene polymorphisms on proton pump inhibitor, amoxicillin, and levofloxacin triple therapy for eradication of Helicobacter pylori. Med Sci Monit 2017; 23: 2701–2707.
15. Rogan PK, Svojanovsky S, Leeder JS. Information theory-based analysis of CYP2C19, CYP2D6 and CYP3A5 splicing mutations. Pharmacogenetics 2003; 13(4): 207–218. doi: 10.1097/01.fpc.0000054078.64000.de.
16. Food and Drug Administration (FDA). Table of pharmacogenomic biomarkers in drug labeling. [online]. Available from: https://www.fda.gov/downloads/Drugs/ScienceResearch/UCM578588.pdf.
17. Hillman L, Yadlapati R, Whitsett M et al. Review of antireflux procedures for proton pump inhibitor nonresponsive gastroesophageal reflux disease. Dis Esophagus 2017; 30(9): 1–14. doi: 10.1093/dote/dox054.
18. Suchopár J, Prokeš M. Polypragmazie a lékové interakce. Vnitř Lék 2011; 57(9): 755–759.
19. Červený P. Inhibitory protonové pumpy: přehled a porovnání základních údajů. Remedia 2009; 9(6): 432–437.
20. Gregar J, Šimková I, Urbánek K. Lékové interakce inhibitorů protonové pumpy. Klin Farmakol Farm 2011; 25(4): 188–191.
21. Sugimoto M, Furuta T. Efficacy of esomeprazole in treating acid-related diseases in Japanese populations. Clin Exp Gastroenterol 2012; 5: 49–59. doi: 10.2147/CEG.S23926.
22. Červený P. Rabeprazol. Remedia 2013; 23(4): 243–247.
23. Li MJ, Li Q, Sun M et al. Comparative effectiveness and acceptability of the FDA-licensed proton pump inhibitors for erosive esophagitis: A PRISMA-compliant network meta-analysis. Medicine (Baltimore) 2017; 96(39): e8120. doi: 10.1097/MD.0000000000008120.
24. Dean L. Omeprazole therapy and CYP2C19 genotype. In: Pratt V, McLeod H, Dean L (eds). Medical Genetics Summaries.. National Center for Biotechnology Information 2012. [online]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK100895/.
25. Tang HL, Li Y, Hu YF et al. Effects of CYP2C19 loss-of-function variants on the eradication of H. pylori infection in patientstreated with proton pump inhibitor-based triple therapy regimens: a meta-analysis of randomized clinical trials. PLoS One 2013; 8(4): e62162. doi: 10.1371/journal.pone.0062162.
26. Serrano D, Torrado S, Torrado-Santiago S et al. The influence of CYP2C19 genetic polymorphism on the pharmacokinetics/- pharmacodynamics of proton pump inhibitor-containing Helicobacter pylori treatments. Curr Drug Metab 2012; 13(9): 1303–1312.
27. NCBI. dbSNP short genetic variations. Reference SNP (refSNP) Cluster Report: rs4244285. [online]. Available from: https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=4244285.

28. Buzková H, Pechandová K, Danzig V et al. Lipid-lowering effect of fluvastatin in relation to cytochrome P450 2C9 variant alleles frequently distributed in the Czech population. Med Sci Monit 2012; 18(8): CR512–CR517. doi: 10.12659/MSM.883272.
29. NCBI. dbSNP short genetic variations. Reference SNP (refSNP) Cluster Report: rs12248560. [online]. Available from: https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=12248560.
30. Lewis JP, Stephens SH, Horenstein RB et al. The CYP2C19*17 variant is not independently associated with clopidogrel response. J Thromb Haemost 2013; 11(9): 1640–1646. doi: 10.1111/jth.12342.
31. Franciosi JP, Mougey EB, Williams A et al. Association between CYP2C19*17 alleles and pH probe testing outcomes in children with symptomatic gastroesophageal reflux. J Clin Pharmacol 2018; 58(1): 89–96. doi: 10.1002/jcph.977.
32. Martínek J, Lukáš M. Inhibitory protonové pumpy – up to date. Gastroent Hepatol 2011; 65(6): 331–342.
33. Furuta T, Sugimoto M, Shirai N et al. CYP2C19 pharmacogenomics associated with therapy of Helicobacter pylori infection and gastro-esophageal reflux diseases with a proton pump inhibitor. Pharmacogenomics 2007; 8(9): 1199–1210. doi: 10.2217/14622416.8.9.1199.

Článek vznikl z prostředků poskytnutých LF MU juniorskému výzkumníkovi Petře Bořilové Linhartové a s podporou grantu GAČR GB14-37368G.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.
The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.
The Editorial Board declares that the manuscript met the ICMJE „uniform requirements" for biomedical papers.

Doručeno/Submitted: 24. 1. 2018
Přijato/Accepted: 30. 3. 2018

Mgr. Petra Bořilová Linhartová, Ph.D.
Ústav patologické fyziologie
LF MU
Kamenice 5
625 00 Brno
plinhart@med.muni.cz

Attachment 4 Article ”Drugs and dosage forms as risk factors for dental caries”

Čes. slov. Farm. 2017; 66, 103–106

PŘEHLEDY A OBDORNÁ SDĚLENÍ

Léčiva a lékové formy jako rizikové faktory pro zubní kaz

Drugs and dosage forms as risk factors for dental caries

Petra Bořilová Linhartová • Lydie Izakovičová Hollá

Došlo: 20. dubna 2017 / Přijato: 8. května 2017

Souhrn

Zubní kaz je multifaktoriální onemocnění, které i přes řadu preventivních opatření zůstává nejrozšířenější infekční chorobou na světě. Náchylnost nebo odolnost jedince k/proti zubnímu kazu je důsledkem interakce rizikových nebo ochranných faktorů, genetické predispozice, nevhodného životního stylu spojeného s úrovní vzdělání, behaviorálními a socioekonomickými faktory. Cílem přehledového článku je upozornit na rizika spojená s užíváním některých léčiv a lékových forem ve vztahu ke vzniku a rozvoji zubního kazu. Dle mechanismu působení v tomto procesu je lze rozdělit na ty, které 1. ovlivňují vývoj zubu a hlavně skloviny, 2. poškozují přímo nebo nepřímo strukturu zubu a/nebo 3. narušují protektivní schopnosti sliny a způsobují dysbiózu orální mikroflóry. V přehledu jsou pak zhodnoceny i současné možnosti terapie a prevence zubního kazu pomocí léčiv, potravinových doplňků a podpůrných prostředků.
Závěr článku se věnuje doporučením pro předcházení nežádoucím účinkům farmakoterapie v dutině ústní, a to pomocí preventivních opatření a/nebo zvážení délky léčby pro dosažení optimálního poměru mezi benefity a riziky. Jelikož je téma publikace mezioborového charakteru, informace o prevenci v rámci zlepšení orálního zdraví v populaci mohou využít nejen farmaceuti, kteří by primárně měli na rizika spojená s farmakoterapií pacienty upozornit, ale také zubní lékaři, pediatři/praktičtí lékaři a široká veřejnost.
Klíčová slova: zubní kaz • prevence • léčivo • léková forma • nežádoucí účinek

Summary

Dental caries is a multifactorial disease which, despite a series of preventive measures, remains the most common infectious disease worldwide. Susceptibility or resistance to caries may be a result of the presence of risk or protective factors, genetic predisposition, inappropriate lifestyle associated with education, behavioural and socioeconomic factors. The aim of this review is to highlight the risks associated with the use of some drugs and dosage forms in relation to the formation and development of dental caries. Drugs are classified by the mechanism of their action in this process, into those that 1. influence the tooth development, especially enamel, 2. directly or indirectly damage the tooth structure and/or 3. disrupt the protective function of saliva, causing oral microflora dysbiosis. The review article assesses the current possibilities in the treatment and prevention of dental caries using drugs, dietary supplements and supporting measures. The conclusion of the study deals with recommendations for prevention of adverse effects of drug therapy in the oral cavity, through preventive measures and/or by considering the duration of the treatment to achieve an optimal balance between benefits and risks. Since the topic of the study is of an interdisciplinary character, information on prevention within the oral health improvement in the population can be used not only by pharmacists, who should primarily warn patients about the risks associated with pharmacotherapy, but also dentists, paediatricians/general practitioners and the public.
Key words: dental caries • prevention • drug • dosage form • adverse effect

Mgr. Petra Bořilová Linhartová, Ph.D. (✉) • L. Izakovičová Hollá
Ústav patologické fyziologie LF MU
Kamenice 5, 625 00 Brno
e-mail: plinhart@med.muni.cz
Stomatologická klinika LF MU a FN u sv. Anny v Brně

Etiologie zubního kazu v dočasné a stálé dentici

Zubní kaz raného dětství (ECC – early childhood caries) i zubní kaz ve stálé dentici patří k onemocněním, která postihují tvrdé tkáně zubu, mohou vést k jeho ztrátě a v krajních případech až k rozsáhlému systémovému zánětu (sepsi). Prevalence zubního kazu je v populaci vysoká i přesto, že etiopatogeneze je do značné míry známá a existuje řada preventivních doporučení.
Za hlavní příčinné agens jsou považovány patogenní bakterie zubního plaku (*Streptococcus mutans*, *Actinobacillus* sp., *Lactobacillus* sp.), jejichž koncentrace bývá zvýšená po narušení rovnováhy složení orálního mikrobiomu. V procesu demineralizace tvrdých tkání zubu: skloviny, dentinu a cementu, hraje roli přítomnost H^+ iontů, a tedy kyselé prostředí, které působí na tyto struktury. V počátečním stadiu tvorby zubního kazu je kyselinám vystavena sklovina pokrývající korunku zubu, která je z asi 93–98 % tvořena krystaly hydroxyapatitu $[Ca_{10}(PO_4)_6(OH)_2]$ a je tak nejtvrdším materiálem v lidském těle.

Ke snížení pH v plaku pod fyziologické rozmezí 6,4–7,0 dochází při příjmu potravy obsahující kyseliny (např. kyselina citronová v ovocných šťávách, H_3PO_4 v kolových nápojích aj.) nebo sacharidy s kariogenním potenciálem. Fermentovatelné sacharidy (sacharóza, glukóza, maltóza, fruktóza, laktóza aj.) mohou sloužit jako substrát pro bakterie, které je metabolizují na organické kyseliny (k. pyrohroznová, k. mléčná, k. octová aj.). Také při refluxu žaludečního obsahu (HCl) do dutiny ústní se zde pH snižuje, dále pak v přítomnosti zánětu (např. zánětu dásní), při příjmu nebo po metabolizaci určitých xenobiotik může docházet ke zvýšení koncentrace H^+ iontů zejména v plaku, ale také ve slině.

Důležitou roli v ochraně proti zubnímu kazu hraje právě slina, a to nejenom svými fyzikálně-chemickými vlastnostmi (množství, viskozita, pufrovací kapacita) ale i přítomností proteinů (imunoglobulin A, lysozym, laktoferin, defenzin, osteopontin aj.), které se podílejí na imunitních reakcích a pomáhají udržovat fyziologické složení orálního mikrobiomu. Při optimální produkci sliny, vzhledem k jejím pufrovacím schopnostem a obsahu minerálů (Ca^{2+}, PO_4^-), se redukuje mikroflóra, odstraňují se zbytky jídla ulpívající na povrchu tvrdých tkání zubu, neutralizují se kyseliny v ústech a je zajištěn remineralizační proces. Pokud však pH plaku klesne pod hodnotu 5,5, dochází k demineralizaci povrchových vrstev skloviny.

Rizikové faktory pro vznik/rozvoj zubního kazu

Za rizikové faktory při vzniku a rozvoji zubního kazu jsou nejčastěji považovány: nedostatečná orální hygiena, vysoká frekvence příjmu kariogenních sacharidů v potravě, dysbióza v dutině ústní, autoimunitní onemocnění (např. Sjögrenův syndrom) a také vrozená náchylnost k zubnímu kazu.

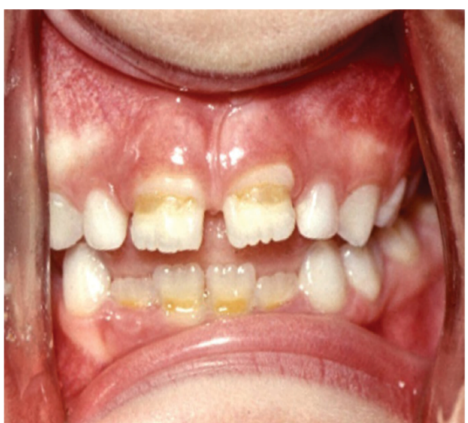
I užívání mnohých léků a doplňků stravy může být spojováno se zvýšeným rizikem rozvoje zubního kazu[1]. Jedná se o léčiva a lékové formy, které dle mechanismů působení můžeme rozdělit do několika skupin:

1. ovlivňují vývoj zubů, a hlavně skloviny
2. poškozují přímo nebo nepřímo struktury zubu
3. a/nebo narušují protektivní schopnosti sliny a vedou k dysbióze v dutině ústní

Léčiva ovlivňující vývoj zubu

Běžné chemikálie (Pb, Hg, anorganické fluoridy, tabák, polycyklické aromatické uhlovodíky) a fyzikální agens (trauma) mohou negativně ovlivnit zuby, a to jak v průběhu embryogeneze, tak i po jejich erupci v dutině ústní[2]. Také užívání některých léčiv může být spojeno s patologickým vývojem zubů, změnou jejich morfologie a struktury, nebo s narušením správného vývoje imunitního systému dítěte. Je však nutné podotknout, že užívání léčiv u gravidních a kojících žen a dětí je v současnosti přísně regulováno.

Asi nejznámějším příkladem negativního vlivu léčiva v tomto kontextu je užívání tetracyklinů, jejichž nežádoucím účinkem je mimo jiné poškození zubní skloviny tvarově i žlutohnědým zabarvením a vznikem tzv. „tetracyklinových zubů" (obr. 1). Na míru závažnosti poškození zubů pak má velký vliv nejenom období, po které se léčivo užívalo (perinatálně, nebo postnatálně – působí však jen v období amelogeneze, tj. cca do 8–10 let věku), ale i jeho dávka a délka léčby.



Obr. 1. Poškození skloviny stálých řezáků po podávání tetracyklinových antibiotik v raném dětství (fotografie z archivu Dětského oddělení Stomatologické kliniky, Fakultní nemocnice u sv. Anny v Brně)

V souvislosti s podáváním léčiv ze skupiny antikonvulziv se nedávno prokázalo, že děti jim prenatálně vystavené mají zvýšené riziko vzniku bílých skvrn na zubech dočasné i stálé dentice a hypoplazie skloviny u dočasných zubů[3]. U dětí, které užívaly během prvních 4 let svého života antiastmatika, byly také nalezeny vývojové vady skloviny, a to u prvních stálých stoliček[4]. Pochopitelně i užívání chemoterapeutik s cytotoxickými vlastnostmi při protinádorové terapii u dětí je spojováno s poruchami vývoje zubu, ať už se jedná o změny v jejich tvaru, počtu, nebo o anomálie skloviny[5].

Uvažuje se také o negativním vlivu kyseliny acetylsalicylové na správný vývoj skloviny dítěte. Ve studii od Nazir et al. byly sledovány zuby novorozených králíků, jejichž matkám byl během těhotenství dlouhodobě podáván aspirin. U novorozených mláďat došlo k hypomineralizaci (Ca^{2+}, Mg^{2+}) řezáků a stoliček[6]. I když se u člověka obdobný proces dosud nezpozoroval, opatrnost je na místě a ani užívání volně prodejných léčiv u těhotných nebo kojících matek a dětí by nemělo probíhat bez porady s lékařem a/nebo lékárníkem.

Léčiva a lékové formy poškozující přímo nebo nepřímo struktury zubu

K léčivům a lékovým formám ***přímo*** poškozujícím zubní sklovinu (ve formě erozí) se mohou řadit ta, která:

• snižují pH v dutině ústní

Při užívání práškových inhalátorů pacienty s astmatem je prášek s účinnou látkou částečně zachycen již v dutině ústní, a protože některá antiastmatika (kortikosteroidy,

$beta_2$-agonisté) jsou kyselé povahy, snižují v ní pH[7] a mohou tak podporovat demineralizační proces skloviny zubů především při dlouhodobé terapii.
Také mnoho antibiotických přípravků je kyselé povahy a ovlivňují tak pH v dutině ústní, pokud jsou podávány perorálně, např. ve formě sirupů dětem. Kritických hodnot pH optimálních pro rozpouštění hydroxyapatitu ve sklovině dosahují např. antibiotické přípravky s kyselinou klavulonovou[8].

K léčivům a lékovým formám ***nepřímo*** poškozujícím zubní sklovinu se mohou řadit ta, která:

- **obsahují kariogenní sacharidy**

Různá léčiva (antibiotika, antitusika, analgetika, antihistaminika aj.), ale i doplňky stravy (vitaminové preparáty) jsou dětem batolecího věku podávána v podobě sirupů obsahujících sacharidy s kariogenním potenciálem, aby jejich užívání bylo pro dítě přijatelné. Častý příjem takové lékové formy může být důvodem ke zvýšené prevalenci ECC. Proto by při jejím opakovaném či dlouhodobém užívání měla být hygiena dutiny ústní zvláště pečlivá a měla by být dodržována i další preventivní opatření. Již před přibližně 40 lety byla publikována studie zabývající se rozvojem zubního kazu u dětí předškolního věku, které pravidelně po dobu alespoň 6 měsíců užívaly léčiva ve formě sirupů. U dětí, které přijímaly sirupy s obsahem sacharózy, byla zaznamenána podstatně vyšší kazivost zubů, a dokonce i častější výskyt zánětu dásní oproti kontrolní skupině. Proto se tehdy navrhovalo, aby léky podávané dětem v této formě neobsahovaly sacharidy, nebo pouze sacharidy nekariogenní[9].
U dospělých mohou být příkladem lékových forem s obsahem cukru, a tudíž potenciálně zvyšujících riziko zubního kazu, žvýkací tablety (např. antacida), pastilky nebo granule. Pomocnými látkami v případě homeopatických granulí jsou glukóza a laktóza, což jsou sacharidy s nejvyšším kariogenním potenciálem[10, 11].*

- **jsou příčinou nauzey**

Léčiva (antidepresiva, anticholinergika, antiastmatika, sedativa, estrogeny aj.), jejichž nežádoucím účinkem je kyselý reflux ze žaludku do horní části trávicího traktu v důsledku relaxace svalů dolního jícnového svěrače nebo nesnášenlivosti léčby, můžeme označit za rizikový faktor pro vznik erozí a rozvoj zubního kazu.

Léčiva narušující protektivní schopnosti sliny a spojená s dysbiózou v dutině ústní

Slinné žlázy jsou inervovány sympatickým (adrenergním) a parasympatickým (cholinergním) nervovým systémem, který ovlivňuje produkci slin. Jelikož slina hraje důležitou roli v ochraně před zubním kazem, dlouhodobé užívání léčiv, u kterých je nežádoucím efektem poškození slinných žláz, zpomalení toku sliny a/nebo změna jejích vlastností, může být rizikovým faktorem pro toto onemocnění. Slina nemůže plnit svou funkci v procesu remineralizace zubní tkáně a udržování rovnováhy ve složení orální mikroflóry.
Suchost úst (xerostomie) je nežádoucím účinkem celé řady léčiv. Za taková jsou považována některá léčiva ze skupiny anticholinergik, antimuskarinik, antidepresiv, antihypertenziv, sympatolytik, $beta_2$-agonistů, anxiolytik, sedativ, opioidů a kanabinoidů, antihistaminik, nesteroidních analgetik a protizánětlivých léčiv, antiparkinsonik, inhibitorů protonové pumpy, cytotoxik, retinoidů, inhibitorů proteáz, diuretik, myorelaxancií, antiepileptik a dalších[12].
S užíváním antiepileptika fenytoinu, antihypertenziva nifedipinu a imunosupresiva cyklosporinu a dalších léčiv je spojován výskyt hyperplazie gingivy, která má negativní vliv na orální hygienu a zvyšuje riziko orálních infekcí[13].
V posledních letech se uvažuje také o významu kandidózy při vzniku a rozvoji zubního kazu; výsledky recentní studie u předškolních dětí dokládají, že vysoká koncentrace kandid ve slinách a přítomnost specifických druhů (*Candida albicans* a *C. dubliniensis*) souvisí se závažností ECC[14]. Dlouhodobé užívání antibiotik může mít vliv na rozvoj kvasinkové infekce na sliznicích dutiny ústní. Dalšími léčivy, jejichž užívání je považováno za rizikový faktor pro rozvoj orální kandidózy, jsou imunosupresiva a systémové nebo inhalační (lokální) kortikosteroidy[15, 16].

Léčiva a doplňky stravy ve vztahu k terapii a prevenci zubního kazu

Z výsledků metaanalýz vyplývá, že antimikrobiální léčba pouze dočasně redukuje množství *S. mutans* v dutině ústní. Neexistují důkazy o tom, že by působení antibiotik mělo protektivní vliv na vznik a rozvoj ECC[17]. Orální bakterie získávají rezistenci na léčiva v současné době používaná a je zde tedy neustálá potřeba vývoje nových preparátů[18].
Ačkoliv se při terapii a prevenci ECC používají různá léčiva, doplňky stravy a podpůrné prostředky, jejich účinek byl shledán v tomto kontextu malý nebo nedostatečný. Efektivitě léčby ECC se věnuje práce od Twetman a Dhar, ve které jsou shrnuty poznatky o vlivu podávání preparátů s fluoridem (past, gelů, tablet, kapek), xylitolu, chlorhexidinu, jódovaného povidonu, probiotických bakterií, remineralizačních činidel (CPP – kasein-fosfo-peptidu), tmelů, dočasných náhrad a významu konzervační (záchovné) stomatologické péče při redukci prevalence ECC[19]. Výsledky poukazují na potřebu vysoce kvalitního experimentálního i klinického výzkumu, na jejichž základech by se dala navrhnout účinnější preventivní opatření[20].

*Kariogenní potenciál těchto sacharidů může být v různých potravinách (např. mléce, mléčných výrobcích a dalších) vyvážen přítomností proteinů a minerálních látek s protektivními vlastnostmi ve vztahu k zubnímu kazu, což ve výsledku znamená nízké riziko nebo dokonce ochranu před vznikem a rozvojem zubního kazu při jejich konzumaci.

Závěr

Pacienti, kteří jsou vystaveni farmakoterapii spojené s potenciálním rizikem vzniku a rozvoje zubního kazu, by měli být o tomto faktu informováni, především pak při častém nebo dlouhodobém podávání příslušných léčiv. Nejohroženější skupinou jsou lidé s poškozenou sklovinou zubu, ať už v důsledku vývojové vady zubů nebo například po bělení zubů, a děti předškolního i školního věku.

Ačkoliv existuje klesající trend výskytu zubního kazu u dětí, Česká republika má nepochybně potenciál pro další zlepšení orálního zdraví v populaci při realizaci adekvátních preventivních opatření. Dle Světové zdravotnické organizace (WHO) je zubní kaz z hlediska léčby čtvrtým nejdražším onemocněním na světě. Součástí strategického dokumentu WHO Zdraví 21 je Dlouhodobý program zlepšování zdravotního stavu obyvatelstva ČR, kdy jedním z cílů je, aby alespoň 80 % dětí do věku 6 let bylo bez zubního kazu v dočasné dentici. Ve věku 12 let by děti měly mít v průměru maximálně 1,5 KPE (zub kariézní, s výplní nebo extrahován) ve stálé dentici[21].

V rámci prevence se doporučuje:

- Po užití preparátů obsahujících kyseliny nebo po zvracení si bezprostředně nečistit zuby (čištění zubů je vhodné až za cca 40–60 minut), pouze dutinu ústní vypláchnout vodou. Pacienti by měli používat měkké zubní kartáčky a zubní pasty s nízkou abrazivitou[22].
- Po podávání orálních preparátů s obsahem kariogenních sacharidů vypláchnout ústa/vypít sklenici vody a provést orální hygienu (případně užít žvýkačku bez cukru nebo s xylitolem), neboť tím jsou odstraněny sacharidy z plaku, hydratují se sliznice a podpoří se tvorba slin. Pokud je to možné, je vhodné neužívat výše zmíněné léky a lékové formy před spaním.
- Pro zmírnění příznaků xerostomie vyplachovat ústa neslazenou minerální vodou, a/nebo použít umělé sliny ve formě spreje, kloktadel, gelů nebo tablet (jsou bez sacharidů a obsahují kalciový pufr). Doporučuje se omezit kofein, je možné užít léčiva stimulující tvorbu slin v případě alespoň částečné funkčnosti slinných žláz (např. parasympatomimetikum pilokarpin).
- Důležitým doporučením je častější frekvence preventivních návštěv zubního lékaře a dentální hygienistky u pacientů, kteří procházejí opakovanou a dlouhotrvající terapii spojenou s užíváním léčiv a lékových forem podporujících vznik a rozvoj zubního kazu.
- V neposlední řadě je vhodné upravit dietní návyky, hydratovat organismus, omezit příjem sladkostí, ovocných šťáv apod. a důsledně se věnovat pravidelné orální hygieně.

Článek vznikl s podporou grantu AZV 17-30439A a z prostředků poskytnutých Lékařskou fakultou MU juniorskému výzkumníkovi Petře Bořilové Linhartové.

Střet zájmů: žádný.

Literatura

1. Drugs that promote dental caries. Prescrire Int. 2015; 24(157), 41–42, 44.
2. **Billings R. J., Berkowitz R. J., Watson G.** Teeth. Pediatrics. 2004; 113(4 Suppl), 1120–1127.
3. **Jacobsen P. E., Henriksen T. B., Haubek D., Ostergaard J. R.** Developmental enamel defects in children prenatally exposed to anti-epileptic drugs. PLoS One. 2013; 8(3), e58213.
4. **Mastora A., Vadiakas G., Agouropoulos A., Gartagani-Panagiotopoulou P., Gemou Engesaeth V.** Developmental defects of enamel in first permanent molars associated with use of asthma drugs in preschool aged children: A retrospective case-control study. Eur. Arch. Paediatr. Dent. 2017; 18(2), 105–111.
5. **Carrillo C. M., Corrêa F. N., Lopes N. N., Fava M., Odone Filho V.** Dental anomalies in children submitted to antineoplastic therapy. Clinics (Sao Paulo) 2014; 69(6), 433–437.
6. **Nazir S., Naqvi S. N. U. H., Ahmed M., Arain A. H., Nazir, N.** Effect of aspirin on the developing teeth of neonates. Int. J. Morphol. 2011; 29(1), 278–285.
7. **Kargul B., Tanboga I., Ergeneli S., Karakoc F., Dagli E.** Inhaler medicament effects on saliva and plaque pH in asthmatic children. J. Clin. Pediatr. Dent. 1998; 22(2), 137–140.
8. **Valinoti A. C., da Costa L. C. Jr., Farah A., Pereira de Sousa V., Fonseca-Gonçalves A., Maia L. C.** Are pediatric antibiotic formulations potentials risk factors for dental caries and dental erosion? Open Dent. J. 2016; 10, 420–430.
9. **Roberts I. F., Roberts G. J.** Relation between medicines sweetened with sucrose and dental disease. Br. Med. J. 1979; 2(6181), 14–16.
10. **Chu J., Zhang T., He K.** Cariogenicity features of Streptococcus mutans in presence of rubusoside. BMC Oral Health. 2016; 16(1), 54.
11. **Assaf D., Steinberg D., Shemesh M.** Lactose triggers biofilm formation by Streptococcus mutans. Int. Dairy J. 2015; 42, 51–57.
12. **Miranda-Rius J., Brunet-Llobet L., Lahor-Soler E., Farré M.** Salivary secretory disorders, inducing drugs, and clinical management. Int. J. Med. Sci. 2015; 12(10), 811–824.
13. **Trackman P. C., Kantarci A.** Molecular and clinical aspects of drug–induced gingival overgrowth. J. Dent. Res. 2015; 94(4), 540–546.
14. **Lozano Moraga C. P., Rodríguez Martínez G. A., Lefimil Puente C. A., Morales Bozo I. C., Urzúa Orellana B. R.** Prevalence of Candida albicans and carriage of Candida non-albicans in the saliva of preschool children, according to their caries status. Acta Odontol. Scand. 2017; 75(1), 30–35.
15. **Patil S., Rao R. S., Majumdar B., Anil S.** Clinical appearance of oral candida infection and therapeutic strategies. Front. Microbiol. 2015; 6, 1391.
16. **Jainkittivong A., Kuvatanasuchati J., Pipattanagovit P., Sinheng W.** Candida in oral lichen planus patients undergoing topical steroid therapy. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 2007; 104(1), 61–66.
17. **Li Y., Tanner A.** Effect of antimicrobial interventions on the oral microbiota associated with early childhood caries. Pediatr. Dent. 2015; 37(3), 226–244.
18. **Kalesinskas P., Kačergius T., Ambrozaitis A., Pečiulienė V., Ericson D.** Reducing dental plaque formation and caries development. A review of current methods and implications for novel pharmaceuticals. Stomatologija. 2014; 16(2), 44–52.
19. **Twetman S., Dhar V.** Evidence of effectiveness of current therapies to prevent and treat early childhood caries. Pediatr. Dent. 2015; 37(3), 246–253.
20. **Hajishengallis E., Parsaei Y., Klein M. I., Koo H.** Advances in the microbial etiology and pathogenesis of early childhood caries. Mol. Oral. Microbiol. 2017; 32(1), 24–34.
21. **Ministerstvo zdravotnictví ČR.** Zdraví pro všechny v 21. Století, 2002. http://www.mzcr.cz/Verejne/dokumenty/zdravi-pro-vsechny-v-stoleti_2461_1101_5.html (8. 4. 2017).
22. **Bartlett D. W.** The role of erosion in tooth wear: aetiology, prevention and management. Int. Dent. J. 2005; 55(4 Suppl 1), 277–284.

Attachment 5 Article ”Relationship between breastfeeding and severe early childhood caries”

Retrospektivní klinická studie
Retrospective Clinical Study

Vztah mezi kojením a výskytem závažného kazu raného dětství

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 3,
s. 59–67

(Původní práce – retrospektivní klinická studie)

Relationship between Breastfeeding and Severe Early Childhood Caries

(Original Article – Retrospective Clinical Study)

Bořilová Linhartová P.[1, 2], Kukletová M.[1], Izakovičová Hollá L.[1, 2]

[1]Stomatologická klinika LF MU a FN u sv. Anny, Brno
[2]Ústav patologické fyziologie LF MU, Brno

SOUHRN

Úvod a cíl: Výsledky výzkumů a názory na kojení ve vztahu k rozvoji kazu raného dětství (ECC) jsou často kontroverzní. Cílem naší práce bylo analyzovat stav chrupu českých dětí s dočasnou dentice v závislosti na době, po kterou byly kojeny, na stravovacích návycích a na úrovni jejich orální hygieny.
Metody: Do této retrospektivní studie jsme zařadili 424 dětí české národnosti z Olomouckého a Jihomoravského kraje, jejichž rodiče podepsali informovaný souhlas a zúčastnili se dotazníkového šetření. Dotazník obsahoval čtyři sady otázek zaměřených na anamnestická data, délku kojení, příjem slazených nápojů a orální hygienu dítěte. Podmínkou pro zařazení do studie bylo prořezání minimálně 16 dočasných a nepřítomnost stálého zubu u dítěte celkově zdravého (včetně absence nadváhy nebo obezity). U dětí jsme zaznamenali kpe index (kaz/výplň/extrahovaný zub) a stav gingivy. Hodnocení dat bylo provedeno s využitím statistického softwaru Statistica v. 13.2.
Výsledky: Mezi 230 chlapci a 194 dívkami průměrného věku 4,0 ± 1,1 let (± směrodatná odchylka) bylo 149 dětí s intaktní dočasnou denticí (kpe = 0) a 275 dětí se závažným ECC (sECC, kpe ≥ 6). U dětí se sECC rodiče v dotazníku častěji uvedli, že dítěti podávají slazené nápoje a začínají mu čistit zuby až od prvního roku věku, než rodiče dětí s intaktní denticí (89,9 % vs. 65,0 %, p < 0,0001, a 80,5 % vs. 40,7 %, p < 0,0001). Kojeno bylo celkem 90,7 % dětí, z toho 52,2 % déle než šest měsíců, a 5,6 % (23 dětí) dokonce déle než 24 měsíců. Po stratifikaci dětí do tří skupin podle doby, po kterou byly kojeny (I. skupina: ≤ 6, II. skupina: 6 a více až ≤ 24, III. skupina: > 24 měsíců), jsme nezaznamenali statisticky významný rozdíl v úrovni orální hygieny mezi dětmi v jednotlivých skupinách, ale v parametru podávání slazených nápojů ano (p < 0,05). Rodiče 77,3 % dětí ve III. skupině uvedli, že s čištěním zubů začali nejdříve po dovršení prvního roku života dítěte; 86,4 % z nich přitom podávalo svým dětem slazené nápoje, což sumárně odpovídá nejrizikovějšímu chování. Dále jsme zjistili, že děti ve II. skupině mají statisticky významně nižší riziko rozvoje sECC oproti dětem v I. skupině (p < 0,001) nebo dětem ve III. skupině (p < 0,01). Pomocí korelační matice jsme analyzovali chování rodičů a nalezli jsme behaviorální vzorec související s orálním zdravím jejich dětí.
Závěr: V etiopatogenezi ECC hraje roli mnoho faktorů; z našich výsledků se kojení dětí v rozmezí šest až 24 měsíců jeví jako protektivní faktor tohoto infekčního onemocnění. Podávání slazených nápojů a pozdní nástup orální hygieny jsme naopak asociovali se sECC. S kojením delším než dva roky je nejvíce spjato rizikové chování matek/osob pečujících o dítě, které může vést k rozvoji sECC.
Klíčová slova: ***kaz raného dětství – gingivitida – kpe index – kojení – mateřské mléko – orální hygiena – výživa – dočasná dentice***

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 3,
s. 59–67

SUMMARY

Introduction and aim: Results of research and opinions on breastfeeding in relation to the development of early childhood caries (ECC) are often controversial. The aim of this study was to analyze the state of teeth of Czech children with primary dentition depending on the time for which they were breastfed, eating habits and the level of their oral hygiene.
Methods: This retrospective study comprised 424 children of Czech nationality from the Olomouc and South Moravian regions, whose parents signed informed consent and participated in the questionnaire survey. The questionnaire contained four sets of questions focused on anamnestic data, duration of breastfeeding, intake of sweetened soft drinks and child's oral hygiene. A prerequisite for inclusion in the study was eruption of at least 16 primary and the absence of a permanent tooth in the generally healthy child (also without overweight or obesity). In children, we recorded dmft index (decay/missed/filled teeth) and gingival condition. Data evaluation was performed using statistical software Statistica v. 13.2.
Results: Of the total set of 424 children (230 boys and 194 girls; mean age ± standard deviation: 4.0 ± 1.1 years), 149 were children with intact primary dentition (dmft = 0) and 275 children with severe ECC (sECC, dmft ≥ 6). In children with sECC, the parents reported in the questionnaire that they served the children more often sweetened beverages and started cleaning their teeth at the age of one year versus the parents of children with the intact dentition (89.9% vs. 65.0%, $p < 0.0001$, and 80.5% vs. 40.7%, $p < 0.0001$). 90.7% of children were breastfed, 52.2% of them longer than six months and 5.6% (23 children) even longer than 24 months. After stratification of children to three groups according to the duration of breastfeeding (Group I: ≤ 6, Group II: 6 < and ≤ 24, Group III: > 24 months, no statistically significant difference in the level of oral hygiene in the children in the individual groups was recorded in contrast to the parameter of serving sweetened beverages ($p < 0.05$). Parents of 77.3% children in Group III reported they started cleaning their children's teeth only after one year of age; 86.4% of them served sweetened liquids to their children, this summarily corresponds to the most risk behavior. Further, we found that the children in Group II had statistically significantly lower risk of the sECC development than the children in Group I ($p < 0.001$) or children in Group III ($p < 0.01$). Behavior of parents was analyzed using a correlation matrix and a behavioral pattern related to oral health of their children was found.
Conclusion: Many factors play a role in the etiopathogenesis of ECC; our results suggest that breastfeeding of children in the duration of 6 to 24 months appears to be a protective factor for this infectious disease. On the contrary, serving sweetened drinks and late start of oral hygiene was associated with sECC. Breastfeeding longer than two years is connected with risk behavior of mothers/caregivers which can result in the development of sECC.
Keywords: *early childhood caries – gingivitis – dmft index – breastfeeding – human milk – oral hygiene – diet – primary dentition*

Čes. Stomat., roč. 118, 2018, č. 3, s. 59–67

ÚVOD

Zubní kaz je multifaktoriálně podmíněné onemocnění indukované kariogenními bakteriemi dentálního plaku (biofilmu), respektive kyselinami, které produkují při metabolizaci fermentovatelných sacharidů. Při jeho vzniku dochází k demineralizaci skloviny a později i dentinu; u neléčených případů až k zánětu pulpy a dalším komplikacím. Protektivní roli v patogenezi zubního kazu sehrává slina, a to nejenom proto, že redukuje množství plaku a zbytků potravy v dutině ústní (objem sliny, rychlost toku a viskozita), ale také díky jejím kvalitativním vlastnostem (pufrační kapacita, koncentrace minerálů a imunoprotektivních proteinů).

Zubní kaz raného dětství (early childhood caries, ECC) je definován přítomností jednoho nebo více kazů (nekavitovaných nebo kavitovaných lézí), zubů s výplní nebo chybějících kvůli zubnímu kazu v jakémkoli dočasném zubu u dítěte ve věku šesti let nebo mladšího. U dětí mladších než tři roky je kaz na hladkém povrchu zubu považován za závažný ECC (severe ECC, sECC). Ve věku od tří do pěti let je za stav sECC považován výskyt jednoho nebo více kavitovaných, zubů s výplní na hladkém povrchu nebo chybějících kvůli zubnímu kazu v oblasti dočasných frontálních zubů horní čelisti, nebo je-li u dítěte určen index kaz/výplň/extrakce (kpe index) ≥ 4 (věk tři roky), ≥ 5 (věk čtyři roky), nebo ≥ 6 (věk pět let) [1].

Názory na kojení ve vztahu k rozvoji ECC jsou často kontroverzní, což dokazují i závěry různých metaanalýz [4, 14, 17]. Především noční kojení podle libosti dítěte staršího jednoho roku a kojení dítěte do jeho vyššího věku jsou považovány za rizikové fak-

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 3,
s. 59–67

tory vzniku ECC [9]. Americká asociace pro dětskou stomatologii (AAPD) dokonce doporučuje v rámci prevence ECC, aby děti s prořezanými dočasnými zuby a v době, kdy již přijímají jinou stravu obsahující sacharidy, nebyly kojeny podle libosti [2]. Na druhou stranu je posilována představa pozitivního významu kojení v rovině psycho-emocionálního a kognitivního vývoje dítěte [6] a i samotného mateřského mléka, díky jeho výživovým a imunoregulačním vlastnostem [11, 16]. Podle doporučení Světové zdravotnické organizace (WHO) je ideální, aby kojenci do věku šesti měsíců přijímali pouze mateřské mléko (a vitamin D), poté aby byla zaváděna tuhá strava a v kojení dítěte matka pokračovala do jeho dvou let věku nebo déle [19].

Vzhledem k nejasné představě o benefitech a rizicích, které představuje kojení pro orální zdraví/nemoc u předškolních dětí, bylo cílem naší práce analyzovat stav chrupu českých dětí s dočasnou denticí v závislosti na době, po kterou byly kojeny, na stravovacích návycích a na úrovni jejich orální hygieny.

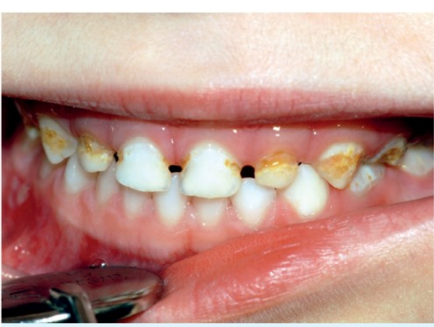
0 – 20 %
21 – 40 %
41 – 60 %
61 – 80 %
81 – 100 %

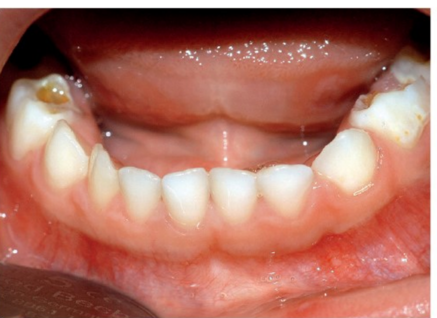
Obr. 1 Grafické znázornění výskytu zubního kazu v dočasné dentici u dětí se sECC (škála definuje procento dětí, které mají postižen zubním kazem daný zub)

METODIKA

V rámci retrospektivní studie jsme od května 2017 do února 2018 vyšetřili 490 dětí předškolního věku z Olomouckého a Jihomoravského kraje a zaznamenali jsme stav jejich chrupu, konkrétně index kpe (hodnotili jsme pouze kavitované léze, tj. kpe D_{3-4}) a gingivy (zánět nepřítomen/přítomen). Podmínkou pro zařazení do studie bylo, aby dítě bylo české národnosti, mělo prořezaných minimálně 16 dočasných zubů (a žádný stálý zub), mělo normální porodní i aktuální váhu, nemělo žádné celkové onemocnění (vrozenou vadu, diabetes mellitus, astma apod.). Oslovili jsme rodiče bez ohledu na jejich socioekonomický status a úroveň vzdělání. Před zařazením do studie rodiče všech dětí podepsali informovaný souhlas, který byl schválen Etickou komisí FN u sv. Anny v Brně, a vyplnili dotazník obsahující čtyři sady otázek zaměřených na anamnestická data, délku kojení, příjem slazených nápojů a orální hygienu dítěte.

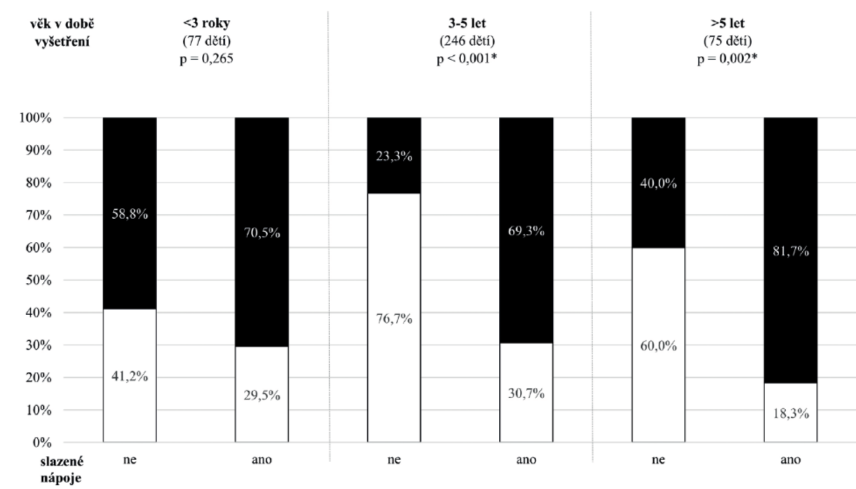
Do studie jsme vybrali pouze 424 dětí, které měly intaktní dentici (kpe = 0) nebo sECC (kpe ≥ 6), a to z důvodu porovnání extrémních fenotypů. Sledovali jsme závislost stavu chrupu a dalších parametrů na době, po kterou bylo dítě kojeno. Pro statistickou analýzu jsme děti rozdělili do tří skupin: na ty,





Obr. 2a, b Ilustrativní snímky dítěte s dočasnou denticí postiženou sECC (fotografie z archivu dětského oddělení Stomatologické kliniky FN u sv. Anny, Brno)

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 3,
s. 59–67



Graf 1 Význam slazených nápojů na vznik sECC ve skupinách dětí podle věku v době vyšetření (hodnoceno Fisherovým exaktním testem)

které nebyly kojeny nebo byly kojeny ≤ 6 měsíců (I. skupina), a na ty, které byly kojeny > 6 měsíců a současně ≤ 24 měsíců (II. skupina). Navíc jsme vyčlenili i děti, které byly kojeny déle než 24 měsíců (III. skupina). Z důvodu komparace našich výsledků s jinými studiemi jsme vytvořili ještě druhý model, kdy děti kojené maximálně 12 měsíců jsme zařadili do skupiny A a děti kojené déle do skupiny B.

Hodnocení dat jsme provedli s využitím statistického softwaru Statistica v. 13.2.

Tab. 1a Frekvence výskytu sECC u dětí podle délky doby, po kterou byly kojeny (hodnoceno Fisherovým exaktním testem)

	Děti s intaktní denticí	Děti se sECC
Jak dlouho bylo dítě kojeno (měsíce) medián [25% a 75% kvartil]	12 [6-18]	8 [3-18]
I. skupina	50 (25,6 %)	145 (74,4 %)
II. skupina	89 (46,8 %)	101 (53,2 %)#,##
III. skupina	5 (21,7 %)	18 (78,3 %)

Pozn. Informace o tom, jak dlouho bylo dítě kojeno, byla uvedena u 408 dětí.
#v porovnání s I. skupinou (p < 0,001)
##v porovnání se III. skupinou (p < 0,01)
I. skupina: ≤ 6, II. skupina: 6 < a ≤ 24, III. skupina: > 24 měsíců, kdy byly děti kojeny

Tab. 1b Frekvence výskytu sECC u dětí podle délky doby, po kterou byly kojeny (hodnoceno Fisherovým exaktním testem)

	Děti s intaktní denticí	Děti se sECC
Skupina A	89 (30,8 %)	200 (69,2 %)
Skupina B	55 (46,2 %)	64 (53,8 %)#

Pozn. Informace o tom, jak dlouho bylo dítě kojeno, byla uvedena u 408 dětí.
#v porovnání se skupinou A (p < 0,01)
skupina A: ≤ 12, skupina B: > 12 měsíců, kdy byly děti kojeny

VÝSLEDKY

Ve sledované kohortě byly děti průměrného věku 4,0 ± 1,1 let (± směrodatná odchylka), 149 dětí mělo intaktní denticí (kpe = 0) a 275 dětí trpělo sECC (kpe ≥ 6). Zastoupení obou pohlaví (54,0 % chlapců a 46,0 % dívek) bylo rovnoměrné i s ohledem na stav jejich chrupu (49,7 % chlapců a 50,3 % dívek s intaktní denticí vs. 56,4 % chlapců a 43,6 % dívek se sECC, p > 0,05). Stejně tak jsme nezaznamenali rozdíl v mediánech [25% a 75% kvartil] porodní váhy mezi dětmi s intakní denticí a dětmi se sECC (3400 g [3000–3700 g] vs. 3350 g [3000–3620 g], p > 0,05). Refluxem, častým ublinkáváním nebo zvracením trpělo 10,6 % dětí, významně častěji se to týkalo dětí s intaktní denticí (19,4 % u dětí s kpe = 0 vs. 5,9 % děti s kpe ≥ 6, p < 0,0001).

Nalezli jsme pozitivní asociaci mezi indexem kpe a gingivitidou, kdy děti s gingivitidou měly medián kpe indexu 11 [9–14] a děti se zdravou dásní měly medián kpe = 0 [0–0] (p < 0,001, (hodnoceno

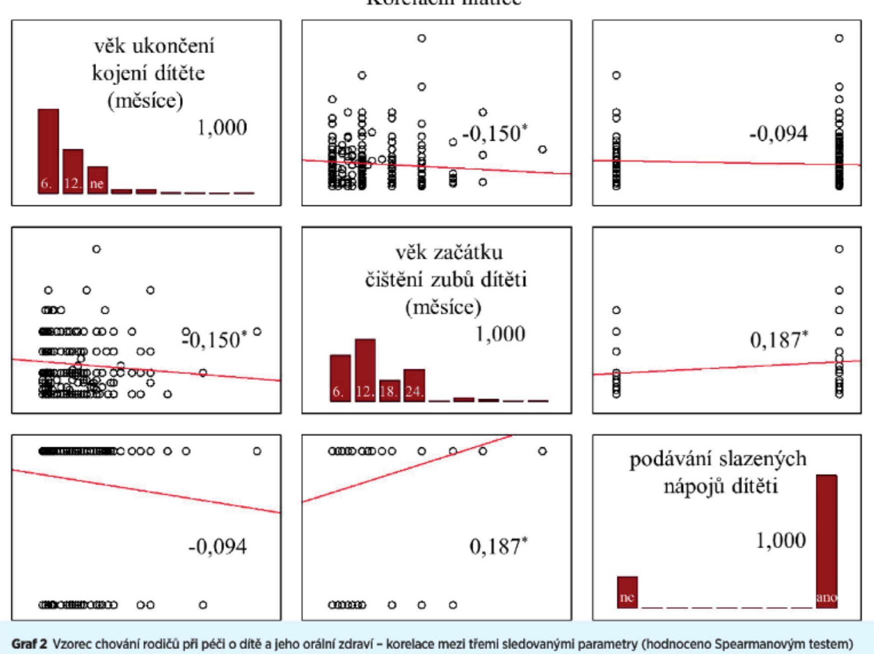
ČESKÁ STOMATOLOGIE
ročník 118,
2018, 3,
s. 59–67

Tab. 2 Sumární přehled (procento zastoupení dětí s intaktní denticí i se sECC) podle tří sledovaných parametrů (hodnoceno Fisherovým exaktním testem)

	Děti s intaktní denticí			Děti se sECC			Všechny děti		
	počet dětí	věk začátku čištění zubů později než ve 12 měsících	podávání slazených nápojů dítěti	počet dětí	věk začátku čištění zubů později než ve 12 měsících	podávání slazených nápojů dítěti	počet dětí	věk začátku čištění zubů později než ve 12 měsících	podávání slazených nápojů dítěti
I. skupina	48 (34,3 %)	11 (22,9 %)	32 (66,7 %)	139 (54,1 %)	113 (81,3 %)	128 (92,1 %)	187 (47,1 %)	124 (66,3 %)	160 (85,6 %)
II. skupina	88 (62,9 %)	43 (48,9 %)†	57 (64,8 %)	100 (38,9 %)	80 (80,0 %)	86 (86,0 %)	188 (47,4 %)	123 (65,4 %)	143 (76,1 %)†
III. skupina	4 (2,8 %)	3 (75,0 %)#	2 (50,0 %)	18 (7,0 %)	14 (77,8 %)	17 (94,4 %)	22 (5,5 %)	17 (77,3 %)	19 (86,4 %)
Celkem	140 (100 %)	57 (40,7 %)	91 (65,0 %)	257 (100 %)	207 (80,5 %)*	231 (89,9 %)*	397 (100 %)	264 (66,5 %)	322 (81,1 %)

Pozn. Informace o všech parametrech zahrnutých v této tabulce byla uvedena u 397 dětí.
***v porovnání s dětmi s intaktní denticí (p < 0,01)**
#v porovnání s I. skupinou (p < 0,001)
†v porovnání s I. skupinou (p ≤ 0,05)
I. skupina: ≤ 6, II. skupina: 6 < a ≤ 24, III. skupina: > 24 měsíců, kdy byly děti kojeny

Kruskalovým-Wallisovým testem). Ve skupině dětí se sECC byly zubním kazem nejčastěji postiženy horní střední řezáky a první moláry v horní i dolní čelisti. Na druhou stranu méně než 20 % dětí se sECC mělo kariézní řezáky nebo špičáky v dolní čelisti (obr. 1, obr. 2a,b).



Graf 2 Vzorec chování rodičů při péči o dítě a jeho orální zdraví – korelace mezi třemi sledovanými parametry (hodnoceno Spearmanovým testem)

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 3,
s. 59–67

U 35,0 % dětí s kpe = 0 rodiče uvedli, že jim dosud nepodávali slazené nápoje, což při porovnání s frekvencí pouze 10,1 % u dětí se sECC představuje statisticky významný rozdíl (p < 0,0001). Celkově však více než 80 % všech rodičů podává svým dětem slazené nápoje. Mezi skupinami dětí rozdělených podle toho, zda jim rodiče podávají slazené nápoje, nebo nikoliv, jsme nalezli statisticky významný rozdíl v procentuálním zastoupení dětí se sECC, a to u dětí ve věku tří až pěti let i u dětí starších než pět let v době vyšetření (p < 0,001, p = 0,002; graf 1). Nejčastěji podávanými slazenými nápoji jsou čaj (68,2 %), šťáva/voda se sirupem (23,4 %), džus (15,6 %) a mléko (12,3 %), zatímco nejméně často je podávána slazená minerální voda (1,3 %).

Rodiče v průměru začali dětem čistit zuby ve věku 16,3 ± 7,1 měsíců, zuby dětem čistí v 76,1 % případů dvakrát denně. U dětí se sECC rodiče častěji než rodiče dětí s intaktní denticí uvedli, že začali svým dětem čistit zuby až od prvního roku věku (80,5 % vs. 40,7 %, p < 0,0001).

Kojeno bylo 90,7 % dětí (99 % i v noci), z toho 52,2 % déle než šest měsíců, a 5,6 % (23 dětí) dokonce déle než 24 měsíců. Frekvence dětí nekojených byla podobná mezi dětmi s kpe = 0 a s kpe ≥ 6 (p > 0,05) a nezjistili jsme také vztah mezi kojením v noci a sECC (62,8 % dětí s kpe = 0 a 54,5 % dětí s kpe ≥ 6, p > 0,05). Ukázalo se však, že děti ze II. skupiny mají statisticky významně nižší riziko rozvoje sECC než děti z I. skupiny (p < 0,001) a i než děti ze III. skupiny (p < 0,01, tab. 1a). Výsledky analýzy provedené dle druhého modelu potvrdily tento trend, neboť u dětí déle kojených (skupina B) jsme zaznamenali nižší prevalenci sECC než u dětí nekojených, nebo kojených kratší dobu (skupina A, tab. 1b).

Nezaznamenali jsme rozdíl v tom, kolik rodičů začalo s orální hygienou po prvním roce života svého dítěte mezi dětmi v I. a II. skupině a ani v tom, kolika procentům dětí jsou podávány slazené nápoje mezi dětmi v I. a III. skupině (p > 0,05). Dětem z II. skupiny rodiče signifikantně méně často podávají slazené nápoje než dětem z I. skupiny (p < 0,05). Nicméně rodiče 77,3 % dětí kojených déle než dva roky uvedli, že s orální hygienou začali až po dovršení prvního roku života dítěte a 86,4 % z nich podává svým dětem slazené nápoje, což sumárně odpovídá nejrizikovějšímu chování mezi skupinami. Dokonce více než polovina rodičů dětí s intaktní denticí uvedla, že začala s čištěním zubů v době mezi prořezáním prvního zubu a dovršením prvního roku dítěte, ale u těch dětí, které byly kojeny déle než do dvou let věku, toto neplatilo (tab. 2).

Negativní vztah jsme zaznamenali mezi věkem dítěte, ve kterém bylo ukončeno kojení, a kdy bylo započato s čištěním zubů (p = 0,003). Statisticky významná byla korelace mezi podáváním slazených nápojů dítěti a věkem, kdy mu rodiče poprvé vyčistili zuby; v tomto případě se jednalo o lineární závislost (p < 0,001, graf 2).

DISKUSE

ECC je jedno z nejčastějších chronických onemocnění v dětství a je spojen s významným zhoršením kvality života dítěte. Kromě sociálních aspektů má toto onemocnění vliv i na schopnost přijímání potravy, na celkový zdravotní stav i na stálý chrup. U mnoha dětí je nutné provést stomatologické ošetření (obvykle mnohočetné extrakce) v celkové anestezii, což je mimo jiné finančně nákladné [10]. V mnoha vyspělých zemích je diagnóza ECC považována za zanedbání péče o dítě [3]; i přesto stále existuje představa, že zdraví dočasných zubů není podstatné, což vede k nedostatečné péči o dutinu ústní dětí [7].

Lokalizace zubních kazů u studované skupiny dětí se sECC logicky odpovídá již známým zjištěním. Dominantní postižení horních řezáků zubním kazem bylo v minulosti popsáno termínem „medové zuby“. Na druhou stranu zuby ve frontálním úseku dolní čelisti, které jsou nejvíce v kontaktu se slinou a jsou chráněny před zubním kazem, byly ve sledovaném vzorku zubním kazem zasaženy u méně než 20 % dětí se sECC. Rovněž společný výskyt gingivitidy a sECC je očekávatelný, u těchto dětí je evidentně zanedbaná orální hygiena a nahromaděný zubní plak obsahuje nejen kariogenní bakterie, ale také mikroorganismy s negativním vlivem na parodontální tkáně, které jsou příčinným faktorem zánětu dásní.

Podávání slazených nápojů dětem a nedostatečná orální hygiena jsou známými rizikovými faktory vzniku ECC; naše výsledky toto zjištění potvrzují. Navzdory varováním a všeobecně proklamovaným výživovým doporučením slazené nápoje dětem nepodává méně než 20 % rodičů. Navíc, podle naší osobní zkušenosti, i když rodiče uvedli, že dětem slazené nápoje nepodávají, tak i těmto dětem jsou v předškolních vzdělávacích zařízeních slazené nápoje nabízeny. Zjistili jsme, že u dětí ve věku vyšším než tři roky v době vyšetření, kterým jsou podávány slazené nápoje, se významně častěji vyskytuje sECC oproti dětem, jejichž rodiče jim slazené nápoje nepodávají. Na druhou stranu u mladších dětí nejsou rozdíly ve frekvenci sECC mezi skupinami podle příjmu slazených nápojů. Z toho vyplývá, že podávání slazených nápojů je rizikovým faktorem pro zubní kaz především v dlouhodobém horizontu a že

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 3,
s. 59–67

na vznik zubního kazu v dočasné dentici mají vliv také jiné faktory, například orální hygiena i genetické predispozice. Pro detailnější analýzu by bylo potřeba provést prospektivní studii, kdy by rodiče uvedli, od kolika let dítěti začali slazené nápoje podávat a v jaké frekvenci takto činí. Alarmující je i průměrný věk, kdy rodiče začínají čistit zuby svým dětem. První zub/y se začínají prořezávat obvykle mezi čtvrtým až šestým měsícem věku a kompletní dočasnou dentici dítě má většina dětí do 30 měsíců věku, tudíž začít s orální hygienou v 16 měsících věku nebo později je jednoznačně nedostatečné. Zřejmě to souvisí i s úrovní orální hygieny rodičů, což je potenciálně další rizikový faktor (který jsme nesledovali), protože mohou infikovat dítě svými patogenními orálními bakteriemi. Ideální je, pokud rodiče zuby dítěte začínají čistit od prvního prořezaného zubu, dítě vedou ke správným návykům, jsou mu vzorem a postupně dítěti předávají zodpovědnost za jeho orální zdraví.

Zajímavým výsledkem je zvýšená prevalence refluxu/zvracení/častého ublinkávání u dětí s intaktní dočasnou denticí. Ačkoliv při těchto stavech může docházet k regurgitaci žaludečního obsahu do dutiny ústní, a tím ke snížení pH, lze očekávat, že by takto vznikaly spíše eroze skloviny než zubní kaz. Je možné, že rodiče dětí s intaktní denticí jsou úzkostlivější a i výjimečné ublinkávání uvedli jako časté.

Pozitivní zjištění je podle našeho názoru to, že převážná většina matek své dítě kojila. Pro statistické zpracování se ukázalo výhodou, že zastoupení dětí v I. a II. skupině bylo podobné.

Zjistili jsme, že kojení dětí déle než šest měsíců a méně než 24 měsíců je asociováno s nižším rizikem rozvoje sECC v české populaci. Ve druhém modelu, kdy jsme zvolili hranici mezi dětmi podle toho, zda byly kojeny pouze 12 měsíců nebo delší dobu, se tento trend potvrdil.

Naše výsledky jsou v kontrastu s recentní metaanalýzou [4], kdy bylo kojení dítěte déle než 12 měsíců asociováno s rizikem ECC. V brazilské kohortové studii zahrnující 1303 dětí bylo za rizikový faktor sECC označeno prolongované kojení (déle než 24 měsíců). Avšak autoři doporučili, aby mechanismy vedoucí k rozvoji u déle kojených dětí byly nadále zkoumány [9]. Podobně se k tomu staví i autoři dalších metaanalýz [14, 17], kde bylo podávání mateřského mléka kojencům v porovnání s náhradní kojeneckou výživou spojeno s protekcí před zubním kazem. Statisticky významnou asociaci mezi kojením delším než 12 měsíců a zubním kazem pak navrhují přisoudit jiným kofaktorům, jako je noční krmení, příjem kariogenních potravin/nápojů nebo nedostatečná orální hygiena [14, 17].

Jelikož nejméně byly podávány slazené nápoje dětem z II. skupiny ve srovnání s ostatními, lze ochranu před vznikem/rozvojem zubního kazu přisoudit výsledku synergického efektu výše zmíněných proměnných. Z našeho výzkumu vyplývá, že čím déle bylo dítě kojeno, tím dříve rodiče začali s čištěním zubů, a naopak. Navíc dětem, u kterých bylo započato s orální hygienou již v době prořezání prvních zubů, rodiče spíše nepodávají slazené nápoje, než těm, kterým začínají čistit zuby později.

Nejhorší vzorec chování a přístupu k ústní hygieně dítěte jsme zaznamenali u rodičů dětí, které byly kojeny déle než dva roky. Rodiče těmto dětem podávali slazené nápoje častěji při srovnání s rodiči dětí nekojených nebo kojených do šestého měsíce věku, případně s rodiči dětí kojených více než půl roku a méně než dva roky. I z tohoto usuzujeme, že považovat kojení dětí delší než dva roky za rizikový faktor zubního kazu bez posouzení vlivu dalších faktorů (např. stravy a čištění zubů) není objektivní. Nicméně počet matek, které kojily dítě více než dva roky, je v naší sledované populaci nízký, proto by výsledky vztahující se k prolongovanému kojení měly být posuzovány s opatrností.

Kojení bývá nepříznivě spojováno pouze se vznikem a rozvojem zubního kazu, ne však se vznikem ortodontických anomálií; u kojených dětí je například nižší riziko malokluze než u dětí krmených z láhve [18]. Podle přehledového článku od Perese a kol. [8] je nezbytné pro racionální zhodnocení vztahu kojení a zubního kazu zvýšit nároky na metodologické přístupy studií. To se týká nejenom sjednocení terminologie a vyloučení zkreslujících proměnných (např. nezahrnovat do studií nedonošené děti), ale také použití vhodných analytických nástrojů. V naší studii jsme se řídili tímto schématem, kritické proměnné, které by mohly zkreslit celkové výsledky, jsme proto stanovili jako vylučovací kritéria pro zařazení dítěte do studie.

Ačkoliv psychosociální a behaviorální faktory ve vztahu k ECC byly analyzovány dosud jen v několika studiích [5, 7, 13], je na základě dosažených výsledků možné navrhnout obecné behaviorální teorie, které mohou napomoci porozumět dopadu a účinnosti intervencí. Nicméně tyto behaviorálně-intervenční nástroje by měly být specifické pro jednotlivé populace, jelikož existují rozdíly nejen mezi rozvojovými a vyspělými státy, ale také mezi jednotlivými kulturně-etnickými komunitami.

Domníváme se, že vyšší prevalence sECC u dětí kojených i ve vyšším věku nalezená v jiných studiích nemusí souviset pouze s příjmem mateřského mléka, ale spíše se vzorcem chování rodičů. Matky, které dítě kojí do jeho vyššího věku, nebo osoby pečující

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 3,
s. 59–67

o tyto děti mohou být v průměru nejvíce benevolentní k dodržování výživových a hygienických pravidel.

Mateřské mléko sice obsahuje laktózu, tedy jeden z fermentovatelných sacharidů, ale jeho součástí jsou také minerály a imunoprotektivní proteiny. Tyto proteiny, např. laktoferin, mají často přímou antimikrobiální aktivitu a jejich koncentrace je řádově vyšší, než je tomu ve slině [12, 15]. Proto je kariogenita mateřského mléka diskutabilní, jednoznačně je však méně rizikovým typem výživy než náhradní mléčná kojenecká výživa, která právě tyto proteiny neobsahuje. Nedoporučujeme zubním lékařům ani pediatrům, aby matky od kojení z důvodu potenciálního rizika zubního kazu odrazovali, neboť naše výsledky ukazují spíše na benefity kojení ve vztahu k orálnímu zdraví. Také Peres a kol. [8] navrhují, aby zubní lékaři a další pracovníci, například dentální hygienistky, vzhledem k nesporným celkovým přínosům kojení pro dítě podporovali doporučení WHO. Pro stomatologickou praxi by potenciální riziko zubního kazu spojené s dlouhodobým kojením mělo být součástí individuálního poradenství pacienta, při kterém jsou zohledněny všechny další relevantní faktory, které se na vzniku zubního kazu mohou podílet a kterým by matky měly věnovat adekvátní pozornost.

Pro sledování vztahu nočního kojení a sECC by bylo vhodnější zvolit prospektivní design studie, jelikož matky si často nepamatovaly, do jakého věku dítě v noci kojily. Na otázku v dotazníku, zda dítě kojily v noci, pak matky kojených dětí v 99 % případů odpověděly, že ano, ale bez udání časové informace, která je pro posouzení rizika možnosti vzniku zubního kazu zásadní. Další limitací naší studie je, že i když jsme oslovili rodiče napříč populací, výzkumu se účastnila jen určitá část z nich.

ZÁVĚR

V etiopatogenezi ECC hraje roli mnoho faktorů; kojení dětí v intervalu šest až 24 měsíců se jeví jako protektivní faktor tohoto infekčního onemocnění, zatímco podávání slazených nápojů a pozdní nástup orální hygieny je možné označit za rizikové chování, které může být příčinou rozvoje sECC. Pro stanovení rizika vzniku tohoto multifaktoriálně podmíněného onemocnění dutiny ústní je třeba brát v úvahu všechny faktory a důsledně se věnovat preventivním opatřením. Behaviorální intervence musí v první řadě cílit na matky a osoby pečující o dítě, ale i samotné dítě by mělo být vedeno k zodpovědnosti za své (nejenom) orální zdraví. Při optimální intervenci cílené na danou skupinu obyvatel by se mohly zlepšit znalosti a chování rodičů i dětí, a tudíž i stav jejich dutiny ústní. Výsledky dosažené v naší studii by měly být ověřeny v dalších evropských zemích a následně by měly vést k zavedení vhodného behaviorálně-intervenčního programu.

LITERATURA

1. **American academy of pediatric dentistry.** Definition of early childhood caries (ECC) [online]. 2008. [cit. 25.4.2018]. Dostupné z: http://www.mychildrensteeth.org/assets/2/7/D_ECC.pdf.
2. **American academy of pediatric dentistry.** Policy on early childhood caries (ECC): classifications, consequences, and preventive strategies [online]. 2016. [cit. 25.4.2018]. Dostupné z: http://www.aapd.org/media/Policies_Guidelines/P_ECCClassifications.pdf.
3. **Bhatia, S. K., et al.:** Characteristics of child dental neglect: a systematic review. J. Dent., roč. 42, 2014, č. 3, s. 229–239.
4. **Cui, L., et al.:** Breastfeeding and early childhood caries: a meta-analysis of observational studies. Asia Pac. J. Clin. Nutr., roč. 26, 2017, č. 5, s. 867–880.
5. **Hooley, M., et al.:** Parental influence and the development of dental caries in children aged 0–6 years: a systematic review of the literature. J. Dent., roč. 40, 2012, č. 5, s. 873–885.
6. **Horta, B. L., de Sousa, B. A., de Mola, C. L.:** Breastfeeding and neurodevelopmental outcomes. Curr. Opin. Clin. Nutr. Metab. Care, roč. 21, 2018, č. 3, s. 174–178.
7. **Nelson, S., et al.:** Do baby teeth really matter? Changing parental perception and increasing dental care utilization for young children. Contemp. Clin. Trials, roč. 59, 2017, s. 13–21.
8. **Peres, K. G., et al.:** Breastfeeding and oral health: evidence and methodological challenges. J. Dent. Res., roč. 97, 2018, č. 3, s. 251–258.
9. **Peres, K. G., et al.:** Impact of prolonged breastfeeding on dental varies: a population-based birth cohort study. Pediatrics, roč. 140, 2017, č. 1, e20162943.
10. **Phantumvanit, P., et al.:** WHO global consultation on public health intervention against early childhood caries. Community Dent. Oral. Epidemiol. [v tisku], 2018.
11. **Prentice, S.:** They are what you eat: can nutritional factors during gestation and early infancy modulate the neonatal immune response? Front. Immunol., roč. 8, 2017, s. 1641.
12. **Rai, D., et al.:** Longitudinal changes in lactoferrin concentrations in human milk: a global systematic review. Crit. Rev. Food Sci. Nutr., roč. 54, 2014, č. 12, s. 1539–1547.
13. **Rai, N. K., Tiwari, T.:** Parental factors influencing the development of early childhood carie s in developing nations: a systematic review. Front. Public Health, roč. 6, 2018, s. 64.
14. **Richards, D.:** Breastfeeding up to 12 months of age not associated with increased risk of caries. Evid. Based Dent., roč. 17, 2016, č. 3, s. 75–76.
15. **Skaličková, S., et al.:** Izolace a stanovení laktoferinu z lidských slin. Chem. Listy, roč. 108, 2014, č. 1, s. 56–63.
16. **Temples, H. S., et al.:** Breastfeeding and growth of children in the peri/postnatal epigenetic twins study (PETS): theoretical epigenetic mechanisms. J. Hum. Lact., roč. 32, 2016, č. 3, s. 481–488.
17. **Tham, R., et al.:** Breastfeeding and the risk of dental caries: a systematic review and meta-analysis. Acta Paediatr., roč. 104, 2015, č. S467, s. 62–84.
18. **Thomaz, E. B. A. F., et al.:** Breastfeeding versus bottle feeding on malocclusion in children: a meta-analysis study. J. Hum. Lact. [v tisku], 2018.

19. World Health Organisation. Breastfeeding [online]. 2018. [cit. 25.4.2018]. Dostupné z: http://www.who.int/topics/breastfeeding/en/.

Poděkování

Autoři článku děkují všem zubním lékařům ze Stomatologické kliniky LF MU, Dětské nemocnice FN Brno a soukromých ordinací (MUDr. Lence Žáčkové, MDDr. Dominice Hulové, MDDr. Michaele Bartošové, Ph.D., MDDr. Denise Kavříkové, MDDr. Zuzaně Bundalové, MDDr. Margaritě Rousi, MUDr. Zdeňku Linhartovi), kteří se podíleli na stomatologickém vyšetření dětí. Dále děkujeme prof. RNDr. Ladislavu Duškovi, Ph.D., za konzultace a pomoc při statistickém hodnocení dat.

Článek vznikl s podporou Ministerstva zdravotnictví České republiky, grantu č. AZV 17-30439A.

RNDr. Petra Bořilová Linhartová, Ph.D.
Ústav patologické fyziologie
LF MU
Kamenice 5
625 00 Brno
e-mail: plinhart@med.muni.cz

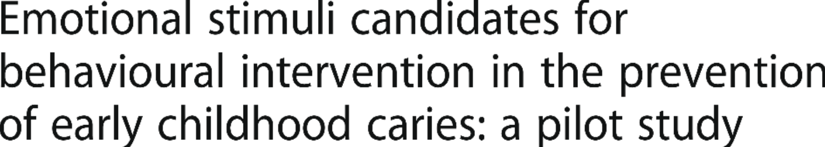
ČESKÁ STOMATOLOGIE
ročník 118,
2018, 3,
s. 59–67

Attachment 6 Article ”Emotional stimuli candidates for behavioural intervention in the prevention of early childhood caries: a pilot study”

Bartosova *et al. BMC Oral Health* (2019) 19:33
https://doi.org/10.1186/s12903-019-0718-4

BMC Oral Health

RESEARCH ARTICLE Open Access



Emotional stimuli candidates for behavioural intervention in the prevention of early childhood caries: a pilot study

Michaela Bartosova[1], Miroslav Svetlak[2], Martina Kukletova[1], Petra Borilova Linhartova[1,3], Ladislav Dusek[4] and Lydie Izakovicova Holla[1,3,5*]

Abstract

Background: Oral diseases, such as early childhood caries (ECC), have a complex etiology with common, behaviour-related risk factors. Appropriately targeted behavioural intervention using effective tools can help to eliminate risk behaviour leading to ECC. The aim of this study was to ascertain which visual stimuli with a supporting text evoke the strongest emotional response in infants' mothers and, therefore, are suitable candidates for inclusion in behavioural interventions within the prevention of ECC.

Methods: Thirty-nine mothers of one-year-old children who filled out an originally designed electronic questionnaire, containing 20 visual stimuli with accompanying texts related to dental caries (10/10 with positive/ negative intended emotional response), were included in this cross-sectional study. The emotional impact of each stimulus in the mothers was evaluated using the Self-Assessment Manikin (SAM) technique, which represents three emotional dimensions: valence, arousal, and dominance.

Results: Each of the stimuli was assessed by the mothers of infants based on its emotional impact. The real emotional response (evaluated according to the median of valence) was in line with the primarily intended response in 90% of cases ($p < 0.05$). The text with a warning evoked a greater emotional response (evaluated according to the median of arousal) in mothers than only the informative instruction ($p < 0.05$). The relationship between arousal and valence ($r = -0.99$; $p < 0.05$) indicates that the more aversive stimuli raise higher arousal. The significant correlation between valence and dominance shows that the more positive the stimuli, the higher feeling of control over the evoked emotion the mothers have ($r = 0.83$; $p < 0.05$), and, on the contrary, the lowest control over emotion is correlated with higher arousal ($r = -0.85$; $p < 0.05$). Generally, mothers rated themselves as in high control of their emotions over the individual stimuli.

Conclusions: This pilot study proved that negative pictorial and text warnings about the risks of developing caries had the potential to evoke strong emotional responses in the mothers of infants. We identified three visual stimuli that could be included in future extensive motivation material in an attempt to affect the preventive behaviour of mothers, and thus the oral health of their infants.

Keywords: Behavioural intervention, Dental caries, Early childhood caries, Prevention, Primary dentition

* Correspondence: holla@med.muni.cz
[1]Clinic of Stomatology, Institution Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Brno, Czech Republic
[3]Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic
Full list of author information is available at the end of the article

© The Author(s). 2019 **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

Background

Dental caries is a multifactorial disease caused mainly by the presence of microbial plaque on the teeth surface related to poor oral hygiene, inappropriate dietary habits, and low tooth surface exposure to fluoride [1]. Despite a number of preventive measures, dental caries remains one of the most widespread infectious diseases in the world. Early childhood caries (ECC) is defined as the presence of one or more decayed (non-cavitated or cavitated lesions [d_1-d_4]), missing (due to caries), or filled tooth surfaces in any primary tooth in a child under the age of six [2]. Severe ECC (s-ECC) is any sign of smooth-surface caries in a child younger than 3 years of age [2]. Based on the etiopathogenesis of ECC, it is possible to define non-modifiable (genetic and socioeconomic) and modifiable (dietary habits and oral hygiene) factors related to this oral disease. It has been suggested that one of the key factors in the etiology and subsequently the prevention of dental caries, is the behaviour of mothers or other caregivers [3, 4]. Despite satisfactory knowledge of the risk factors for dental caries, information alone is not sufficient to change behaviour and a special behavioural intervention is necessary [5].

Emotions are closely linked to the approach and avoidance motivation strategies which represent the fundamental motivational forces governing human behaviour. They can be viewed as "push" forces that predispose to act in a certain way [6]. Each motivated behaviour can be divided into two tendencies by activating two different brain systems: the tendency to approach a pleasant stimulus (reward system) and the tendency to repulse from an aversive stimulus (defence system) [7]. The general aim of behaviour could be defined as an effort of people to move closer to the desired emotional state. In other words, actions in reaction to emotions should lead to the achievement of a more 'good for me' or less 'bad for me' state [8]. In order to evoke the person's motivation to behave in a certain way, the stimulus that is to provoke must be connected with the emotional reaction, in the sense of the basic assessment as to whether it is a good or bad state for the person.

Currently, most information on dental caries prevention is presented as a professional recommendation in a rather emotionally neutral textual form. The "evidence-based" recommendation produces positive emotions in health care providers, for others it is rather neutral and incomprehensible. Although verbal expressions of emotions and feelings in humans are very variable, factor-analytic studies of emotional language have concluded that each stimulus can be placed into a three-dimensional affective space which is defined by: emotional valence, emotional arousal and dominance [9]. These dimensions are closely connected with the motivation of behaviour. The coordinates of the stimulus (e.g., photograph, text, sound, etc.) in the affective space, expressed by valence, arousal and dominance, represent important information about its motivational power.

In this context, searching for effective approaches to motivate people to change risk behaviour or to create health-promoting behaviour, is a major challenge of preventive medicine.

Methods

The aim of this pilot study was to ascertain which visual stimuli with a supporting text evoke the strongest emotional response in infants' mothers and, therefore, are suitable candidates for inclusion in behavioural interventions within the prevention of ECC.

The originally designed electronic questionnaire (Additional files 1 and 2) contained 20 stimuli, which represented the risk factors of dental caries in children or showed the appropriate behaviour of mothers in the care of their children's dentition. The visual stimuli (10 with an intended positive response and 10 with an intended negative response), accompanied by verbal descriptions, warnings or information (instruction), were designed in line with the recommendations of the European Academy of Pediatric Dentistry [1]. The visual stimuli were chosen according to the experience of the clinicians from our out-patients clinic. The most serious conditions were included in aversive pictures. The positive picture set was composed of the most desired aims of prevention of dental caries (pictures 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20).

The emotional impact of these 20 individual stimuli on mothers was evaluated using the Self-Assessment Manikin (SAM) method (Fig. 1) [10]. The SAM graphical scale is a tool for the assessment of the three basic emotional dimensions: valence, arousal, and dominance. The emotional valence (first line in Fig. 1) is a bipolar scale which describes a continuum between polarities that are extremely unpleasant (unhappy manikin on the left) and extremely pleasant (happy manikin on the right). The scale ranges from 1 to 9 (1 extremely unpleasant, 5 neutral, 9 extremely pleasant). The arousal scale (the second line in Fig. 1) represents a continuum which ranges from 1 (calm, relaxed manikin on the left) to 9 (extremely excited, aroused manikin on the right). The dominance scale (third line in Fig. 1) describes the level of subjectively referred sense of control over the emotion evoked by the stimulus. It ranges from 1 (loss of control, manikin on the left) to 9 (full control, manikin on the right). The subject is instructed to evaluate their immediate subjective feeling being evoked by the test material (texts and pictures in our study) across the three afore-mentioned dimensions.

During the period from 04/2017 to 10/2017, 97 mothers of one-year-old children were addressed with a request to join this cross-sectional study. Ninety-seven infants were examined with a dental mirror and probe by one pediatric dentist at the Clinic of Stomatology, Faculty of Medicine, Masaryk University and St. Anne's Faculty Hospital. The

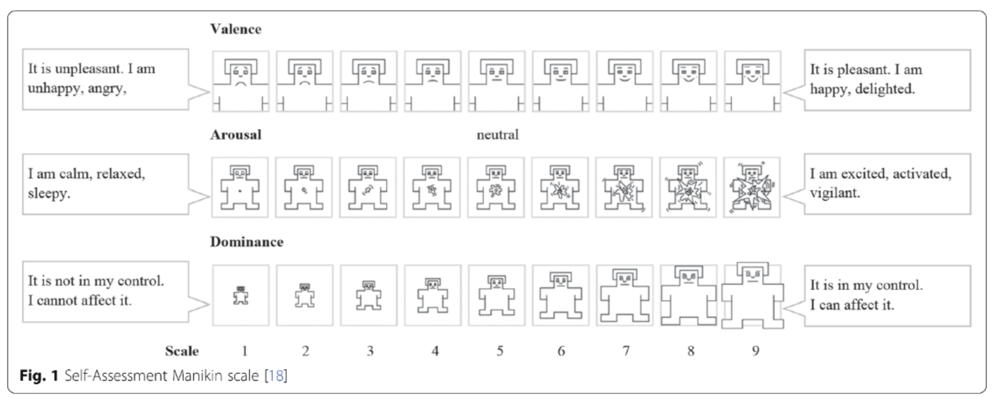


Fig. 1 Self-Assessment Manikin scale [18]

$d_1$mft index was calculated using dental caries (d_1 level) as a cut-off point for the detection of decay. There were no exclusion criteria for mothers and infants. Only 39 mothers signed informed consent forms and agreed to participate in our study. The basic demographic characteristics (age of mothers, education of both parents), and their awareness of caries risk factors and dental caries prevention, i.e. suitable diet, drinking sweet beverages, significance of teeth cleaning/ brushing, suitable teeth brushing techniques, observance of oral health of their children etc., were determined in all of them. Subsequently, after being instructed as to how to record the emotional perception evoked in them by a stimulus on the SAM scale, (see Additional file 1), the mothers were asked to fill out the electronic questionnaire. The link to the electronic questionnaire was sent to mothers' e-mail addresses, the questionnaire was filled out online.

For statistical purposes, the scope of the SAM technique was converted to numbers ranging from 1 to 9. The software Statistica v. 13 (IBM Corporation, 2013) was used for the data analysis. The relationships between the 3 dimensions (valence, arousal, dominance) were computed using Spearman's correlation coefficient (r). For the description of variables the mean and standard deviation (SD), median and interquartile range (IRQ), and minimum and maximum were used. Statistical significance of relationship between categorical variables was tested using Fisher exact test, statistical significance of differences in ordinal scores between groups was tested using Mann Whitney U test. The sample size was planned on the basis of expected width of 95% confidence interval for average score estimate with expected standard deviation of pseudo-continuous scoring scale 1. The acceptable width for the 95% confidence interval of average score estimate was set as 0.7; it corresponds to $N = 35$. The computation of the overall sample size was estimated using previously experienced response rate approximately 30–40% in similar studies [11–13]. The response rate to the questionnaires was estimated to be assumed to be approximately 40%, thus 97 mothers were addressed to reach 35–40 responses.

Results

In this pilot study, 39 mother-child pairs were included. The electronic questionnaire response rate was 40.2%. 35.9% of mothers were in the age group of 21–30 years and 64.1% were in the age group of 31–40 years. 51.3% mothers had higher (university) education. Only four mothers had lower than secondary school education. The mean age ± standard deviation (SD) of the infants was 11.2 ± 2.4 months (median age [interquartile range, minimum-maximum] 10.0 [10.0–12.0, 8.0–20.0] months) with 5.6 ± 3.8 (5.0 [3.0–7.0, 0–16.0]) primary teeth. The examination revealed beginning of caries in three children ($d_1$mft) on all upper incisors, i.e. $d_1$mft = 4, the other children were caries free.

Five mothers reported that they did not use tooth paste for cleaning their infants' teeth, only two mothers served sweetened beverages to their children and none of them dipped a pacifier into something sweet before giving it to their child. Up to 28.2% of mothers cleaned a pacifier in their mouths and even 43.6% of them occasionally or regularly tasted a meal with the same spoon or licked the spoon while feeding the infant.

Each of the stimuli in the electronic questionnaire was defined based on its emotional impact on the infants' mothers. Table 1 shows the statistical evaluations (median and interquartile range) of valence, arousal, and dominance produced by ranking the individual stimuli in the questionnaire. There was a highly statistically significant relationship between the expected and observed emotional result of the visual stimuli (10 negative and 10 positive vs. 8 negative and 12 positive, $p < 0.05$). The real emotional response (evaluated according to the median of valence) was in line with the primarily intended response in 90% of cases. Highly statistically significant difference in the median

Table 1 Ranking of the stimuli in the questionnaire (valence, arousal, dominance)

S	V	T	Text	Median (IQR)			R
				Valence	Arousal	Dominance	
5	N	W	Lack of mother's care for her infant's teeth causes tooth decay.	*1 (1–2)*	*8 (5–9)*	8 (7–9)	N
13	N	W	Neglecting care of your child's teeth leads to serious complications.	*1 (1–3)*	*7 (5–9)*	8 (6–9)	N
7	N	W	Untreated tooth can also endanger your child's life.	*2 (1–3)*	*7 (5–9)*	8 (6–9)	N
15	N	W	Caries hurt children.	*2 (1–3)*	7 (3–8)	7 (5–9)	N
17	N	W	Poor dentition requires anesthesia.	*2 (1–5)*	6 (3.5–8)	8 (5–9)	N
9	N	W	By neglecting regular care at the dentist, you expose your child to an unpleasant treatment.	*3 (2–5)*	6 (3–8)	7 (7–9)	N
3	N	W	Sleeping with a bottle increases the risk of tooth decay.	3 (2–7)	4 (2–7)	9 (7–9)	N
19	N	W	Mother may be a source of bacteria supporting tooth decay in a child.	4 (2–7)	5 (3–7)	7 (5–9)	N
20	P	I	By avoiding kissing baby on the lips, you reduce the risk of transmission of bacteria that cause tooth decay.	6 (3–9)	3 (1–6)	8 (6–9)	P
1	N	I	We do not offer sweetened drinks to children.	7 (4–9)	3 (2–5)	9 (7–9)	P
16	P	I	Preventive examination takes place twice a year.	8 (5–9)	2 (1–5)	9 (8–9)	P
4	P	I	A visit to a dentist can be painless.	8 (5.5–9)	2 (1–5)	7 (6.5–9)	P
11	N	I	Regular dental examinations allow the dentist to detect dental caries in time and treatment is painless.	8 (6–9)	2 (1–4)	9 (8–9)	P
14	P	I	We clean our child's teeth twice a day.	8 (7–9)	2 (1–3)	9 (7–9)	P
2	P	I	Parents must assist their child with care for their teeth till the child is six.	9 (7–9)	2 (1–4)	9 (7–9)	P
6	P	I	At the age of 1 year, child eats from his/her own saucer and with his/her own cutlery.	9 (7.5–9)	2 (1–4)	8 (6.5–9)	P
18	P	I	Regular care protects your child's teeth.	9 (8–9)	1 (1–3)	9 (8–9)	P
8	P	I	Drinking pure water reduces the risk of tooth decay in children.	9 (8–9)	1 (1–2)	9 (8–9)	P
12	P	I	Parents are the ultimate model for children.	9 (8–9)	1 (1–2)	9 (8–9)	P
10	P	I	Regular care protects your child's teeth.	9 (8–9)	1 (1–2)	9 (8–9)	P

The relevant statements are ordered according to their valence, from the most negative to the most positive. *The combination of visual and text stimuli with the highest potential for the use in behavioural interventions.* Numbers in italics represent stimuli assessed by more than 75% of mothers with valence ≤5/ arousal ≥5. S = stimulus in the questionnaire, V = intended visual stimulus, T = text stimulus, R = emotional response of mothers to visual and text stimuli, IQR = interquartile range, N = negative, P = positive, W = warning, I = information

values of valence between groups defined according to the expected negative and positive emotional result was observed (10 intended negative emotional stimuli with median of valence 2 [IQR, 2–4] and 10 intended positive emotional stimuli with valence 9 [IQR, 8–9], $p < 0.05$). The text with a warning evoked a greater emotional response in mothers than only the informative instruction. The significant difference in median values of arousal between groups categorized according to the type of text was found (8 text warnings with median of arousal 6 [IQR, 5–7] and 12 text instructions with arousal 2 [IQR, 1–2], $p < 0.05$).

Stimulus 5 was perceived as the most negative with the lowest valence of 1 and the highest arousal of 8, with 95% of the mothers giving the picture and the description a negative valence (valence less than 5) and 69.2% high arousal. Stimuli 3, 5, 7, 9, 13, 15, 17, and 19 exhibited low valence (median from all texts and pictures ≤5) and can be considered as negative. High arousal (median of all statements ≥5) was recorded only in stimuli 5, 7, 9, 13, 15, 17, and 19. Negative stimuli 5, 7, and 13 including text warnings were assessed by more than 75% of mothers with a valence ≤5 and at the same time with an arousal ≥5 (Fig. 2).

Our results demonstrated that not all stimuli originally intended as negative were perceived by the infants' mothers adversely. Negative stimuli 1 and 11 were evaluated by mothers with valence 7 and 8 (respectively), and thus as positive. However, stimuli 19 and 20 with an intended opposite response were evaluated by mothers as we had presumed, i.e. they evoked a rather neutral valence and a mean value of arousal (Fig. 2).

It is evident that the exposure of mothers to stimuli did not cause a significant loss of control over the evoked emotions (see Table 1). The median value of all responses was higher than 5 in all cases. For this reason, only the results relating to the remaining two subscales (i.e. valence and arousal) are further presented.

All positive images and descriptions generated positive valence and low arousal (Fig. 3). Stimuli 8, 10, 12, and 18 (with median of valence 9 and arousal 1) were evaluated as the most positive ones. The relationship between

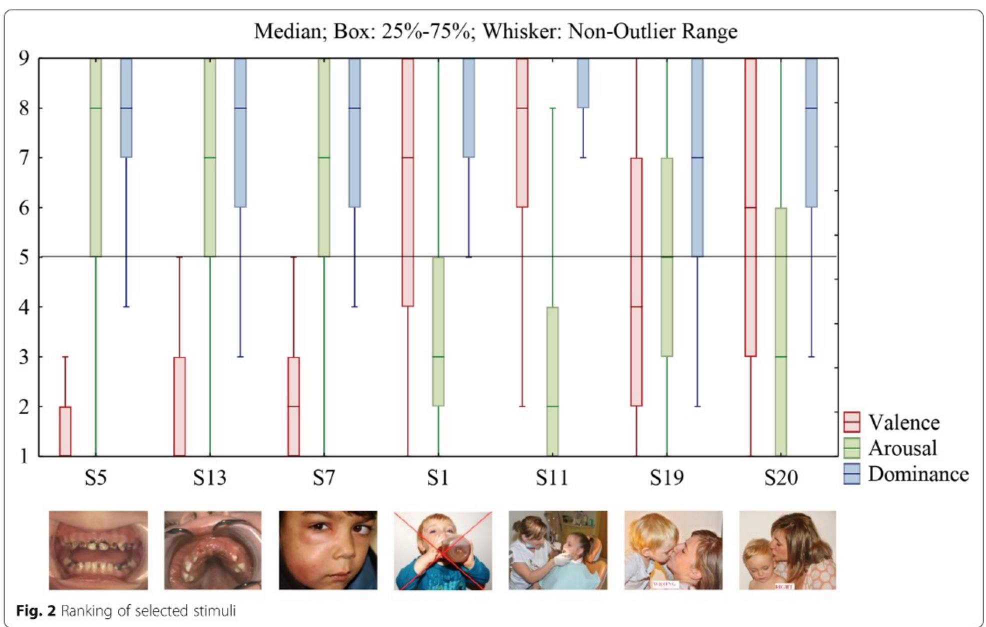


Fig. 2 Ranking of selected stimuli

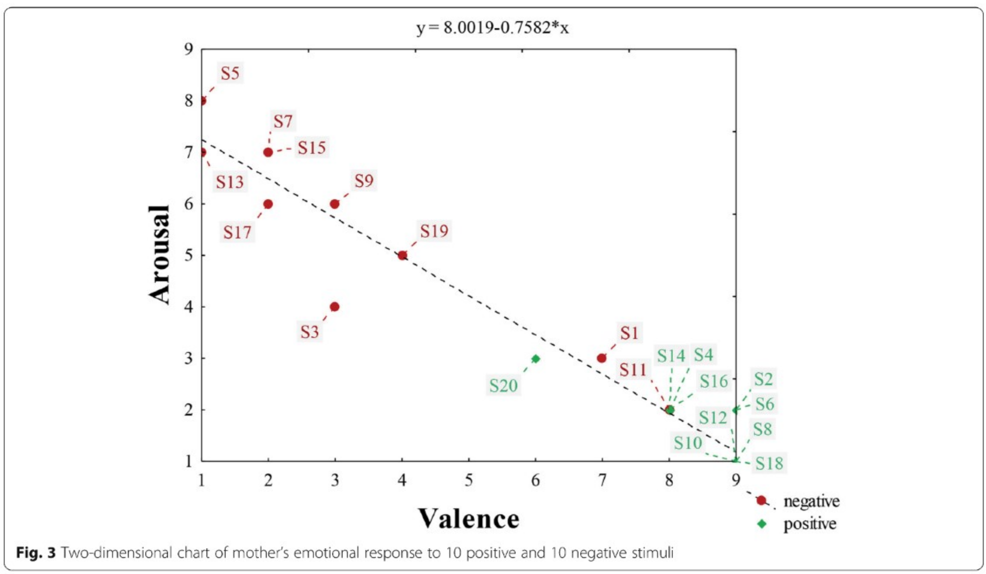


Fig. 3 Two-dimensional chart of mother's emotional response to 10 positive and 10 negative stimuli

arousal and valence ($r = -0.99$; $p < 0.05$) indicates that the more aversive stimuli raise higher arousal. The significant correlation between valence and dominance shows that the more positive the stimuli, the higher feeling of control over the evoked emotion the mothers have ($r = 0.83$; $p < 0.05$), and, on the contrary, the lowest control over emotion is correlated with higher arousal ($r = -0.85$; $p < 0.05$). Generally, mothers rated themselves as in high control of their emotions over the individual stimuli.

Discussion

Several studies have focused on behavioural interventions within prevention of ECC and other oral diseases. Behavioural interventions to reduce caries have been based on a variety of behaviour change theories and approaches – mainly the social cognitive theory and the related health belief model and the theory of planned behaviour, self-determination theory, and motivational interviewing [14, 15].

The preventive programs are primarily aimed at children at primary schools [16]. Programs trying to influence parental behaviour are rare [17]; they mostly affect the frequency of tooth cleaning but do not lead to a change of dietary habits in children [17]. We believe that intervention within prevention of ECC should be targeted at mothers as they play an important role in health behaviour of the whole family, especially infants.

As already mentioned, emotions are an important part of the motivation to change behaviour. Triggering positive or negative emotions associated with problematic behaviour is one of the fundamental processes of this change [18]. The most effective approaches in preventive medicine are those with minimal economic costs and maximum population impact [19]. Searching for stimuli that evoke strong emotions and address the relevant target groups is a key step of each effective preventive intervention.

This also was the aim of the submitted pilot study as the stimulus material for the intervention approach for the Czech population has not been formed and standardized yet. Stimuli 5, 7, and 13 in our questionnaire have the lowest valence and the highest potential to evoke a strong emotional response in the mothers of infants and thus may be used as aversive stimuli within behavioural interventions in the field of oral health. The highest arousal was evoked by a picture of the damaged teeth of a child and the statement "Lack of mother's care for her child's teeth causes tooth decay." The high dominance associated with all stimuli (including stimulus 5) could be explained again by the fact that the experimental group probably consisted of responsible mothers, who visit the dentist regularly and have no reason to worry.

While the negative pictures with warnings were evaluated by mothers as we had presumed, both negative figures with educational texts were evaluated with an unexpectedly high valence. In stimulus 1, this can also be explained by the fact that the crossed-out positive figure does not evoke negative emotions. In stimulus 11, the figure shows a child being treated by a dentist, similarly as in stimuli 4, 9, 16, and 17. We suppose that the mothers of infants in our studied group perceived the dental treatment neutrally and thus these types of figures should be replaced by other images in following studies.

Nevertheless, stimuli 1 and 11 represent an interesting subgroup of pictures with supporting texts in terms of the emotional responses of mothers. The responses to them very likely depend on the behaviour of the mothers and thus constitute a continuum between positive and negative valence reactions. Mothers participating in this study cared for their children's oral health and generally maintained preventive measures. Therefore, they appreciated this information as positive and might be willing to follow the recommendations. Negative responses could be expected from the mothers on the opposite side of the continuum whose behaviour is unhealthy and negative pictures with texts confront them with this fact.

In general, current prevention medicine uses only a limited repertoire of behaviour change techniques [20, 21]. It mostly focuses on providing information on the relationship between behaviour and health or fear appeal. For example, the global prevention campaign on tobacco products is based on aversive visual stimuli and text warnings. However, it has been repeatedly shown that education and intimidation are not enough [14] and that people are more motivated by moving towards positive emotions [22] than by avoiding negative situations. The approach often neglected by a contemporary biomedical model of medicine is based on the premise that rather than avoiding fear it is more natural for people to move towards some appreciated personal values and principles (e.g.,"to be a good mother"). A preventive campaign based on this principle should be the next focus of research into the prevention of tooth decay.

For this reason, positive stimuli were also included in our questionnaire, for example a picture of a mother who is cleaning her teeth with her child and the sentence: "Parents are the ultimate model for children". This sentence was rated by mothers as one of the most positive, but with very low arousal. Finding that positive images are associated with lower arousal agrees with the results of other studies [23]. It is still unclear what role arousal plays in the positive motivation and what its optimal level for the most effective behavioural change should be.

The warning or information about the inappropriateness of kissing a child on the mouth (stimuli 19 or 20) due to the possible transfer of the cariogenic bacteria from the mother's mouth into the child's oral cavity evoked a rather

neutral valence and a mean value of arousal, whereas the interquartile range is greatest at the valence of these stimuli. It probably reflects great ambivalence of mothers to this statement. The picture of mother kissing her child with information about a possible transmission of bacteria has the potential to be used as a positive stimulus in the intervention process.

The main limitation of this study is a low number of probands due to a lack of interest across a group of mothers of infants. The fact that the electronic questionnaire was filled out by the mothers online may have played a role in the low response rate. Mothers were not under pressure to participate. On the other hand, responders should be in emotional stable state when filling out the electronic questionnaire. The final number of the obtained questionnaires generated a sample size which was sufficient for the planned statistical estimates with a relevant confidence interval.

Another problem is the significant impact of selection, especially when mothers with higher motivation and generally better knowledge of risk factors of tooth decay ("conscious mothers") were willing to be involved in the study. There is a high probability that mothers with risk behaviour were not interested in participating in the questionnaire survey. Further research will need to be done on a larger sample of the population, including mothers from risk groups.

Our constant hypothesis is that the stimulus material should firstly impress mothers with a negative warning, thus developing negative emotions in the form of concerns about their child's health, and at the same time offer them a solution as to how to avoid the feared consequences of their behaviour. In the future follow-up study, we intend to create a portfolio of emotionally-charged stimuli (images with texts) and then verify their effectiveness in behavioural intervention. The stimuli material can be a beta version for a motivational interview or a smart phone application which we will test on the Czech population. We can draw inspiration from the studies in the population of the American Indians [24] or studies by Nolen et al. [25]. It will be necessary to determine the percentage of mothers who really have changed their behaviour on the basis of the given prevention method (reduced consumption of sweetened drinks, regular preventive examinations, etc.) and to verify whether the defensive mechanisms such as generalization or rationalization were not activated.

Conclusions

This pilot study proved that negative pictorial and text warnings about risks of developing caries had the potential to evoke strong emotional responses in the mothers of infants. We identified three visual stimuli which could be included in a future extensive motivation material in an attempt to affect the preventive behaviour of mothers, and thus the oral health of their infants. Future research in this area should focus on creating extensive stimulus material and its subsequent verification on a large population.

Additional files

Additional file 1: Instructions for filling out the electronic questionnaire (PDF 124 kb)

Additional file 2: Overview of the stimuli in the electronic questionnaire (S1-S20) (PDF 364 kb)

Abbreviations

d: decay, part of $d_3$mft decay/missing/filled teeth index; ECC: early childhood caries; I: information; IQR: interquartile range; N: negative; P: positive; p: *p*-value; r: correlation coefficient; R: emotional response of mothers to visual and text stimulus; S: stimulus in the questionnaire; SAM: Self-Assessment Manikin; SD: standard deviation; s-ECC: severe form of early childhood caries; T: text stimulus; V: intended visual stimulus; W: warning

Acknowledgements

Not applicable.

Funding

The study was supported by funds from the Faculty of Medicine MU to junior researcher Michaela Bartosova, project MUNI/A/1008/2017, by the Ministry of Health of the Czech Republic, grant no. 17-30439A, and by grant RECETOX Research Infrastructure supported by the Czech Ministry of Education, Youth and Sports (LM2015051) and the European Structural and Investment Funds, Operational Programme Research, Development, Education (CZ.02.1.01/0.0/0.0/16_013/0001761). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

MK, MS and MB designed the study, prepared the questionnaire and drafted the manuscript. MB examined the infants and addressed mothers for participation in the study. MS, PBL, LIH and LD performed the statistical analysis and participated in the interpretation of data. PBL and LIH assisted with reviewing and revising the final manuscript. All authors approved the manuscript as submitted and take full responsibility for the manuscript.

Ethics approval and consent to participate

The study was approved by the Committee for Ethics of the Faculty of Medicine Masaryk University Brno (1G/2017). We confirm that written informed consent was obtained from all participants. The images and photographs in Fig. 2 and Additional file 2 belong to the authors.

Consent for publication

Written consent for publication was obtained from all the infants' caregivers in this manuscript.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details

[1]Clinic of Stomatology, Institution Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Brno, Czech Republic. [2]Department of Psychology and Psychosomatics, Faculty of Medicine, Masaryk University, Brno, Czech Republic. [3]Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic. [4]Institute of Biostatistics and Analyses, Masaryk University, Brno, Czech Republic. [5]RECETOX – Research

Center for Toxic Compounds, Faculty of Science, Masaryk University, Brno, Czech Republic.

Received: 19 April 2018 Accepted: 21 January 2019
Published online: 18 February 2019

References

1. The European Academy of Paediatric Dentistry. Guidelines on Prevention of Early Childhood Caries: an EAPD policy document. In: Policies and guidelines. The European Academy of Paediatric Dentistry. 2008. http://www.eapd.eu/uploads/1722F50D_file.pdf. Accessed 20 Nov 2018.
2. American Academy of Pediatric Dentistry. Policy on early childhood caries (ECC): classifications, consequences, and preventive strategies 2017;39(6):59–61.
3. Kukletova M, Izakovicova Holla L, Musilova K, Broukal Z, Kukla L. Relationship between gingivitis severity, caries experience and orthodontic anomalies in 13-15 year-old adolescents in Brno, Czech Republic. Community Dent Health. 2012;29(2):179–83.
4. Wilson AR, Mulvahill MJ, Tiwari T. The impact of maternal self-efficacy and oral health beliefs on early childhood caries in Latino children. From Public Health. 2017;5:228.
5. Kukletova M, Kuklova J. ECC and the possibilities of prevention [text in Czech, abstract in English]. Prakt Zubn Lek. 2004;52(5):150–6.
6. Frijda NH. Studies in emotion and social interaction. The emotions. New York: Cambridge University press, Paris: Maison de sciences de l'Homme; 1986.
7. Schneirla TC. An evolutionary and developmental theory of biphasic processes underlying approach and withdrawal. In: Jones MR, editor. Nebraska symposium on motivation. Lincoln: University of Nebraska Press; 1959. p. 1–42.
8. Etkin A, Egner T, Kalisch R. Emotional processing in anterior cingulate and medial prefrontal cortex. Trends Cogn Sci. 2011;15(2):85–93.
9. Osgood CE, Suci GJ, Tannenbaum P. The measurement of meaning. 1st ed. Urbana: University of Illinois Press; 1957.
10. Bradley MM, Lang PJ. Measuring emotion: the self-assessment manikin and the semantic differential. J Behav Ther Exp Psychiatry. 1994;25(1):49–59.
11. Ismail A, Nainar SM, Sohn W. Children's first dental visit: attitudes and practices of US pediatricians and family physicians. Pediatr Dent. 2003;25(5):425–30.
12. Grembowski D, Spiekerman C, Milgrom P. Linking mother access to dental care and child oral health. Community Dent Oral Epidemiol. 2009;37(5):381–90.
13. Araia MH, Wilson J, Chakraborty P, Gall K, Honeywell C, Milburn J, Ramsay T, Potter BK. Factors associated with knowledge of and satisfaction with newborn screening education: a survey of mothers. Genet Med. 2012;14:963–70.
14. Albino J, Tiwari T. Preventing childhood caries: a review of recent behavioral research. J Dent Res. 2016;95(1):35–42.
15. Randall CL. On motivational interviewing for oral health promotion: state of the field and future directions. JDR Clin Trans Res. 2018;3(4):376–77.
16. Cooper AM, O'Malley LA, Elison SN, Armstrong R, Burnside G, Adair P, et al. Primary school-based behavioural interventions for preventing caries. Cochrane Database Syst Rev. 2013;5:CD009378. https://doi.org/10.1002/14651858.CD009378.pub2.
17. O'Malley L, Adair P, Burnside G, Robinson L, Coffey M, Pine C. An evaluation of a storybook targeting parental attitudes, intention, and self-efficacy to change their child's oral health behavior. Health Psychol. 2017;36(2):152–9.
18. Prochaska JO, Norcross JC. Systems of psychotherapy: atranstheoretical analysis. 5th ed. Pacific Grove: Brooks/Cole Pub; 2003.
19. Velicer WF, Prochaska JO, Fava JL, Rossi JS, Redding CA, Laforge RG, et al. Using the transtheoretical model for population – based approaches to health promotion and disease prevention. Homeost Health Dis. 2000;40(5):174–95.
20. Abraham C, Michie S. Taxonomy of behavior change techniques used in interventions. Health Psychol. 2008;27(3):379–87.
21. Martino S. Oral health behavioral and social intervention research concepts and methods. J Public Health Dent. 2011;71Suppl 1:S2–S6.
22. Seligman MEP. Authentic happiness: using the new positive psychology to realize your potential for lasting fulfillment. 1st ed. New York: Free Press; 2002.
23. Bradley MM, Lang PJ. Fearfulness and affective evaluations of pictures. Motiv Emot. 1999;23(1):1–13.
24. Batliner TS, Tiwari T, Henderson WG, Wilson AR, Gregorich SE, Fehringer KA, et al. Randomized trial of motivational interviewing to prevent early childhood caries in American Indian children. JDR Clin Trans Res. 2018;3(4):366–75.
25. Nolen SL, Giblin-Scanlon LJ, Boyd LD, Rainchuso L. Development and testing of a smartphone application prototype for oral health promotion. J Dent Hyg. 2018;92(2):6–14.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

Attachment 7 Article ”Commercial tests for the determination of dental caries and periodontal disease risk and development”

2
2019
ROČNÍK 29

RECENZOVANÝ ČASOPIS ČESKÉ STOMATOLOGICKÉ KOMORY

PRVNÍ OSTRAVSKÉ
STOMATOLOGICKÉ FÓRUM

...aby informace přijely za vámi a vy jste nemuseli za nimi...

Odborná diskuze
RIZIKA PŘERUŠENÍ ANTIAGREGAČNÍ A ANTIKOAGULAČNÍ LÉČBY U ZUBNÍCH VÝKONŮ

Jak nás vidí
O STOMATOLOGII A JEJÍCH PERSPEKTIVÁCH S ADAMEM VOJTĚCHEM, MINISTREM ZDRAVOTNICTVÍ

Co vás zajímá
KANCELÁŘ ČSK – SERVIS PRO VÁS 4. ČÁST: KOMUNIKACE

Indexováno: Bibliographia Medica Čechoslovaca, Index Copernicus. ISSN 1210-3381 (Print). ISSN 2571-2411 (Online).

KOMERČNÍ TESTY PRO STANOVENÍ RIZIKA ROZVOJE ZUBNÍHO KAZU A ONEMOCNĚNÍ PARODONTU

Přehledové sdělení

Petra Bořilová Linhartová[1,2], Denisa Kavříková[1], Simona Slezáková[1,2], Martina Kukletová[1], Lydie Izakovičová Hollá[1,2]
1) Stomatologická klinika, Lékařská fakulta Masarykovy univerzity a Fakultní nemocnice u sv. Anny, Brno
2) Ústav patologické fyziologie, Lékařská fakulta Masarykovy univerzity, Brno

O AUTORCE

*RNDr. Petra Bořilová Linhartová, Ph.D., (*1984) absolvovala magisterské studium v oboru Biochemie na PřF MU v Brně a státní rigorózní zkoušku v oboru Imunologie na PřF UK. Doktorské studium ukončila v oboru Fyziologie a patologická fyziologie na LF MU v Brně pod vedením prof. MUDr. Lydie Izakovičové Hollé, Ph.D., a od roku 2010 pracuje na Stomatologické klinice LF MU a FNUSA v Brně. Je odbornou asistentkou a vyučuje několik předmětů na PřF MU a LF MU, vede studenty bakalářských, magisterských i doktorských programů na obou fakultách. V rámci výzkumu se zabývá především molekulárně genetickými aspekty multifaktoriálně podmíněných onemocnění dutiny ústní. Je autorkou 33 odborných článků, z toho 23 prací bylo publikováno v impaktovaných časopisech, její h-index je aktuálně 7. Je recenzentkou 10 mezinárodních časopisů a řešitelkou nebo spoluřešitelkou několika výukových i výzkumných projektů.*

Kontakt:
plinhart@med.muni.cz
Ústav patologické fyziologie
LF MU v Brně
Kamenice 5
625 00 Brno

SOUHRN: Přehledový článek popisuje výhody a nevýhody testů aktuálně dostupných na českém trhu (případně v EU), které jsou zubním lékařům nabízeny k diagnostice zubního kazu a/nebo onemocnění parodontu u dětí i dospělých, k výběru optimálních terapeutických postupů i k ověření efektivity léčby. Testování mohou být prováděna přímo v ordinaci zubního lékaře nebo v externí laboratoři a hodnotí se nejčastěji množství a kvalita sliny, počet vybraných kariogenních a/nebo parodontálních bakterií, případně geneticky podmíněná predispozice pacienta (variabilita v genu pro prozánětlivý cytokin interleukin-1). Existuje několik variant kitů s různou citlivostí a přesností; některé z nich však vyžadují další přístrojové vybavení. V rámci posílení prevence a orální péče mohou být některé testy pro určení individuální náchylnosti k těmto chorobám účinným nástrojem při behaviorální intervenci nejenom u pacientů s rizikovým nálezem.

Klíčová slova: test, slina, orální mikroflóra, prevence, parodontitida, zubní kaz

COMMERCIAL TESTS FOR THE DETERMINATION OF DENTAL CARIES AND PERIODONTAL DISEASE RISK AND DEVELOPMENT

Review article

SUMMARY: The review describes the advantages and disadvantages of commercial tests currently available to dentists on the Czech (or EU) market for diagnosing the dental caries and/or periodontal disease in children and adults, for selection of optimal therapeutic procedures and for veryfying of the treatment efficiency. These tests are intended for dental practice; testing can be performed directly in the dentist's office or in an external laboratory. The saliva quantity and quality, the number of selected cariogenic and/or periodontal bacteria, or the genetic predisposition of the patient (variability in the proinflammatory cytokine interleukin-1 gene) are evaluated. Several variants of kits with different sensitivity and accuracy are available; some of them require additional instrumentation. In the context of strengthening prevention and oral care, several tests

for the determination of the individual susceptibility to these diseases can be an effective tool in behavioral intervention not only in patients with risk finding.
Key words: test, saliva, oral microflora, prevention, periodontitis, dental caries

Bořilová Linhartová P, Kavříková D, Slezáková S, Kukletová M, Izakovičová Hollá L. Komerční testy pro stanovení rizika rozvoje zubního kazu a onemocnění parodontu. LKS, 2019, 29(2): 30–37.

ÚVOD

Onemocnění dutiny ústní u dětí i dospělých jsou aktuálním problémem současné medicíny, proto existuje intenzivní snaha o co nejpřesnější a nejjednodušší **diagnostiku** jejich etiologie, **stanovení optimálního léčebného plánu** dle individuálního nálezu a současně o **posílení preventivních opatření pomocí behaviorální intervence**. Nejčastěji pacienti trpí zubním kazem a/nebo onemocněním závěsného aparátu zubů (1), kdy v etiopatogenezi obou těchto multifaktoriálních onemocnění hrají roli orální bakterie a schopnost organizmu se s jejich přítomností vyrovnat bez poškození vlastních tkání.

Protektivní roli sehrává **slina**, která svým tokem odplavuje zbytky potravy z dutiny ústní a ovlivňuje složení orální mikroflóry. Současně slina obsahuje proteiny s různou funkcí (imunoprotektivní, antimikrobiální, pufrovací atd.) a ionty. Pufrovací systémy, které neutralizují kyselé prostředí v dutině ústní, jsou tři: fosfátový, bílkovinný a bikarbonátový (hydrogenuhličitanový). pH v ústech je ovlivňováno nejenom složením stravy a nápojů (sladkosti, kyselé nápoje apod.), ale také užíváním léčiv (např. inhalační kortikosteroidy) (2), regurgitací kyselého obsahu žaludku do dutiny ústní a/nebo celkovým zdravotním stavem pacienta.

Při vzniku a rozvoji zubního kazu hraje úlohu častá a/nebo nadměrná spotřeba sacharidů (zejména sacharózy) a přítomnost grampozitivního fakultativního anaeroba *Streptococcus mutans* a dalších kariogenních bakterií (*Lactobacillus* sp., *Actinomyces* sp.) (3), které jsou schopny metabolizovat fermentovatelné sacharidy za vzniku organických kyselin zodpovědných za demineralizaci skloviny. Zubní kaz postihuje dočasnou i stálou dentici a orální bakteriální profil je závislý nejenom na typu dentice, ale i na rozsahu kariézního procesu (4).

Jelikož bakterie schopné vyvolat onemocnění soutěží s běžnými orálními symbiotickými druhy v dutině ústní, vede jejich pomnožení k narušení rovnováhy a ke změně složení orálního biofilmu, k tzv. dysbióze. **Dysbiotický stav v dutině ústní** může vést také k onemocnění závěsného aparátu zubu, jedná se převážně o polymikrobiální infekci, avšak bez jednoznačné spojitosti se specifickou bakterií/bakteriemi. Nejčastěji jsou u pacientů s parodontitidou detekovány gramnegativní anaeroby červeného komplexu (*Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*) a oranžového komplexu (*Fusobacterium nucleatum*, *Parvimonas micra*, *Prevotella intermedia*, *Campylobacter rectus*) (5, 6). Z přehledu současné literatury nevyplynuly žádné kvalitativní rozdíly ve složení orálního mikrobiomu mezi pacienty s agresivní (AgP) a s chronickou parodontitidou (CP) (7). *Aggregatibacter actinomycetemcomitans* je sice spojován se zvýšeným rizikem lokalizované AgP (8), především přítomnost vysoce virulentního klonu JP2 v subgingiválním prostoru může sloužit při predikci vzniku a progrese onemocnění, bývá však prokazován pouze u určité malé podskupiny pacientů (9).

Mombelli (10) ve své práci píše, že v současné době neexistuje pro léčbu jakékoliv formy onemocnění parodontu protokol s prokazatelnou nadřazeností, na druhou stranu existují i studie navrhující konkrétní účinné látky nebo postupy pro léčbu pacientů se specifickým mikrobiálním profilem (11, 12). Pokud u pacientů budou definovány klíčové rizikové bakteriální druhy, je možné individuálně přistoupit k jejich eradikaci/potlačení a současně umožnit rekolonizaci příznivé a zdravé flóry, ať už konvenčními metodami, nebo novými biotechnologickými přístupy (13).

Ačkoliv se na výzkumném poli hledají genetické **markery určující individuální náchylnost jedince k zubnímu kazu i k parodontitidě**, výsledky studií jsou často protichůdné a závislé nejenom na typu dentice, ale liší se také mezi různými populacemi. V současné době je variabilita v genu kódujícím prozánětlivý cytokin interleukin-1 (IL-1) považována za jeden z možných faktorů ovlivňujících vznik a rozvoj parodontitidy (14). V naší nedávné studii (15) jsme však u pacientů v české populaci zjistili, že ti s T alelou v místě +3953C/T (rs1143634) v sekvenci *IL-1beta* genu mají menší riziko rozvoje CP; různé haplotypy (kombinace alel) v *IL-1* genovém klasteru mohou ovlivňovat také riziko rozvoje CP u pacientů s/bez diabetes mellitus.

CÍL A METODIKA ANALÝZY TRHU

Cílem přehledového článku bylo shrnout informace o komerčních testech dostupných na českém trhu (eventuálně v EU), které jsou doporučeny k využití ve stomatologii/zubním lékařství. Při analýze trhu jsme postupovali tak, že jsme oslovili firmy nabízející produkty nebo služby v oblasti dentální diagnostiky a vyžádali jsme si od nich nezávazné nabídky a podrobné informace ke komerčním kitům pro testování sliny, orální mikroflóry a/nebo vrozené náchylnosti k zánětlivým onemocněním. Firmy jsme vybrali na základě osobní zkušenosti, doporučení kolegů praktických zubních lékařů a parodontologů, nebo s využitím internetových vyhledávačů.

KOMERČNÍ TESTY PRO STANOVENÍ VLASTNOSTÍ SLINY

Testování sliny lze využít v řadě klinických situací, ať už u pacientů s dentinovou hypersensitivitou, s onemocněním slinných žláz (xerostomie, Sjögrenův syndrom), při posouzení rizika vzniku a rozvoje zubního kazu i parodontitidy. Na druhou stranu je však třeba poznamenat, že měření vlastností sliny se nejeví jako vhodný nástroj pro hodnocení rizika vzniku atrice/abraze (16). Přehled testů pro analýzu sliny a jejich specifikace jsou uvedeny v **tab. I**, kde jsme kromě testů zjišťujících pufrovací kapacitu sliny (CRT buffer Refill 6, Ivoclar Vivadent) a případně i další parametry (Saliva-Check Buffer, GC Europe) zahrnuli také testovací proužky pro určení pH sliny (Hydrion (O67) Urine & Saliva pH Paper, Micro Essential Laboratory, Inc.). Testování pH sliny je založeno na principu kolorimetrické reakce, kdy pomocí barevného indikátoru můžeme identifikovat pH sliny, a to v rozmezí 5,5 až 8,0. Obdobně je stanovována i pufrovací kapacita sliny, která je určena z třístupňové škály: nízká, střední, vysoká.

Ve srovnávacích studiích hodnotících korelaci mezi kolorimetrickými testy typu strip (CRT buffer Refill 6, Ivoclar Vivadent a Saliva-Check Buffer, GC Europe) a laboratorními metodami pro analýzu pufrovací kapacity slin bylo zjištěno, že test Saliva Check Buffer (GC Europe) je přesnější než test CRT Buffer Refill 6 (Ivoclar Vivadent) (17, 18), pomocí testu Saliva Check Buffer (GC Europe) lze stanovit vícero parametrů (rychlost toku, viskozitu a konzistenci nestimulovaných slin, pH pacientovy klidové i stimulované sliny a její množství); navíc je i levnější.

Obecně lze říci, že testování sliny k určení míry rizika vzniku zubního kazu a parodontitidy je založeno na jednoduchých principech a provedení, které je možno zvládnout v čase do 10 minut. Ke všem uvedeným testům je přiložen český návod, není potřeba dalšího přístrojového vybavení a provedení testu je proto možné přímo v ordinaci zubního lékaře nebo dentální hygienistky. Kritickým momentem je vlastní odběr sliny (stimulované i nestimulované), kdy pacient nesmí 1 hodinu před odběrem jíst, žvýkat žvýkačku, kouřit, čistit si zuby a používat ústní vodu, pít by měl pouze čistou vodu. Odečet výsledku je nenáročný a velmi názorný, především pak po předložení výsledku pacientovi může být toto testování vhodným nástrojem pro intervenční proces. Rozhodující pro výběr ideálního testu pro konkrétního pacienta tedy bude cena a/nebo komplexnost výsledku.

Obr. 1

Obr. 1: *Odběr sulkulární tekutiny a zubního plaku ze zubu s největším zánětem u dítěte s dočasnou denticí (fotografie z archivu Dětského oddělení Stomatologické kliniky LF MU a FN u sv. Anny v Brně)*

KOMERČNÍ TESTY PRO STANOVENÍ ORÁLNÍCH MIKROORGANIZMŮ

Determinace vybraných mikroorganizmů osidlujících dutinu ústní je založena na několika základních principech, a to na kultivaci bakterií na selektivních médiích, na imunochromatografické detekci antigenu nebo na identifikaci specifické sekvence genu pro malou podjednotku ribozomální RNA (16S rRNA) pomocí metod využívajících polymerázovou řetězovou reakci (PCR). PCR se již stala standardním diagnostickým a výzkumným nástrojem v parodontologii, jelikož její citlivost a specifičnost umožňuje rychlou a účinnou metodu detekce, identifikace a kvantifikace mikroorganizmů i stanovení genetického profilu pacienta (19).

Přítomnost kariogenních bakterií lze zjistit pomocí testu CRT® bacteria Refill 6 (Ivoclar Vivadent) založeného na kultivaci nebo pomocí imunochromatografického testu Saliva-Check Mutans (GC Europe), viz **tab. II**. Oba testy jsou ekonomicky stejně náročné (cca 450 Kč na 1 test) a vstupním vzorkem je slina pacienta. Test Saliva-Check Mutans (GC Europe) určený pro analýzu *S. mutans* nevyžaduje další přístrojové vybavení a výsledek je znám do 15 minut, zatímco test CRT® bacteria Refill 6 (Ivoclar Vivadent) pro stanovení *S. mutans* a *Lactobacillus* sp. vyžaduje kultivaci vzorku při teplotě 37 °C po dobu 2 dnů. Je možné využít i produkt CRT Intro Pack (Ivoclar Vivadent), který obsahuje testy CRT Buffer Refill 6 a CRT® bacteria Refill 6.

Pro stanovení vícero kariogenních a/nebo parodontálních bakterií lze využít několika komerčních testů. Protože jsou založeny na PCR, je nezbytné použít termocykler a jejich provedení tedy není možné v běžné zubní ordinaci, viz **tab. II**. Vzhledem k vysoké variabilitě bakteriálních genomů je možné, že některé subtypy nemusí být možné těmito testy stanovit.

V České republice lze kontaktovat firmy Protean, GenTrend, HAIN LIFESCIENCE GmbH (distributor v ČR Eurotex, s. r. o.) nebo PřF MU, které analýzu vybraných bakterií nabízejí formou služby. Pro stanovení mikroorganizmů z parodontálního sulku je možné oslovit také firmy v Německu – Carpegen GmbH (test Carpegen® Perio Diagnostik, dříve Meridol® Diagnostik) nebo ve Švýcarsku – IAI AG (iai PadoTest). Italská firma Saccace Biotechnologies pak nabízí k zakoupení sadu (PeriodontScreen Real-TM, v ČR distribuováno firmou Dynex), s jejíž pomocí si ve vlastní laboratoři

s odpovídajícím laboratorním vybavením lze vybrané orální mikroorganizmy vyšetřit (obdobně lze zakoupit i sady od HAIN LIFESCIENCE GmbH).

Ke stanovení je třeba, aby byl odebrán vzorek sulkulární tekutiny a/nebo zubního plaku a přiložena žádanka s identifikací pacienta a lokalizace zubu, ze kterého byl vzorek odebrán. Odběr vzorku se provádí pomocí papírového čepu (ISO 40 nebo 50) ze zubu s největším zánětem, viz **obr. 1**. Nejenom při porovnání výsledků před a po terapii je nutné dodržovat standardizovaný postup odběru, který je uveden v návodu, a používat stejný typ testu. Z externí laboratoře jsou výsledky vyšetření často i s doporučením vhodné antibiotické léčby doručeny nejpozději do 14 dnů.

Kvalitativní analýza je možná při použití všech metodických přístupů, rozdíl je však v možnosti determinace počtu druhů v jedné reakci a v přesnosti (citlivosti) kvantitativního stanovení. Z tohoto úhlu pohledu se jeví molekulárně biologický přístup, konkrétně multiplexová PCR v reálném čase, jako nejvýhodnější, jelikož umožňuje stanovení vícero bakteriálních druhů v jedné reakci a současně poskytuje i informaci o jejich počtu ve vzorku, a to i v procentuálním zastoupení vůči celkovému mikrobiomu (testy od PřF MU). Na základě komplexního mikrobiologického vyšetření lze poté lépe určit míru rizika resorpce závěsného aparátu zubu a kosti nebo zubního kazu. Současně lze tyto testy využít i k analýze bakteriálních komplikací po implantologické léčbě.

Při nálezu určitých parodontálních bakterií ve vyšším počtu je nejvhodnějším postupem odeslat pacienta na specializované pracoviště, kde parodontolog provede detailní klinické i mikrobiologické vyšetření, na jehož základě poté zvolí odpovídající terapii. Navíc parodontolog může při určitých stavech (diagnózy K052, K053, K055, K056) vykázat mikrobiologické vyšetření k úhradě některým zdravotním pojišťovnám (např. VZP), což není v kompetenci praktického zubního lékaře. Uvedené testy se stanovením založeným na principu PCR se používají nejenom v klinické praxi, ale zatím zejména při výzkumu etiopatogeneze onemocnění dutiny ústní (20, 21, 22).

Přínosem testování mikrobiálního profilu pacienta by bylo, pokud by testy odhalily také případnou rezistenci bakterií ke konkrétním antibiotikům. Ačkoliv některé firmy (Protean a GenTrend) průkaz rezistence k beta-laktamovým antibiotikům nabízejí, je nutné poznamenat, že je toto založeno na analýze genů kódujících betalaktamázy v DNA (chromozomální, plazmidová, transpozonová). Pro přesné určení, zda je gen pro betalaktamázu exprimován a bakterie je tedy rezistentní k beta-laktamovým antibiotikům, by měla být provedena i analýza fenotypu (23).

Tabulka I: *Přehled komerčních testů pro stanovení vlastností sliny – dostupných v ČR*

Název testu	Saliva-Check Buffer	CRT buffer Refill 6	Hydrion (067) Urine & Saliva pH Paper
Firma	GC Europe	Ivoclar Vivadent	Micro Essential Laboratory, Inc.
Možnost provést vyhodnocení přímo v ordinaci	ano	ano	ano
Nutnost přístrojového vybavení	ne	ne	ne
Rychlost stanovení	10 minut	5 minut	neprodleně
Vstupní vzorek	slina	slina	slina
Princip stanovení	• kolorimetrie • vizuálně	• kolorimetrie	• kolorimetrie
Cena s DPH	3020 Kč	1099 Kč	69 Kč (399 Kč)
Počet testů v jednom balení	20	6	15 ks (100 ks)
Návod v češtině	ano	ano	ano
Co zjistíme	• rychlost toku • viskozitu a konzistenci nestimulovaných slin, • pH nestimulované i stimulované sliny • množství stimulované sliny • pufrovací kapacita sliny	• pufrovací kapacita sliny	• pH sliny
Výhody	• komplexní analýza • nejpřesnější z testů • názornost	• názornost	• cena za 1 test (cca 5 Kč)
Nevýhody	• cena za 1 test (cca 151 Kč)	• cena za 1 test (cca 183 Kč)	• stanovení pouze pH

Tabulka II: *Přehled komerčních testů pro stanovení kariogenních a parodontálních bakterií – dostupných v ČR*

Název testu	**Saliva-Check Mutans**	**CRT® bacteria Refill 6 #**	**Stoma-Gene® ##**	**VariOr® Dento 7 ###**
Firma	GC Europe	Ivoclar Vivadent	Protean	GenTrend
Možnost provést vyhodnocení přímo v ordinaci	ano	ano	ne	ne
Nutnost přístrojového vybavení	ne	ano (inkubátor)	ano	ano
Rychlost stanovení	15 minut	2 dny	do 2 týdnů	do 5 pracovních dnů
Vstupní vzorek (odběr sliny/ zubního plaku/sulkulární tekutiny)	slina	slina	sulkulární tekutina	sulkulární tekutina
Princip stanovení	imunochromatografie	kultivace	PCR	PCR
Cena s DPH	4560 Kč	2687 Kč	3025 Kč + zdarma odběrový set	1500 Kč + zdarma odběrový set
Počet testů v jednom balení	10	6	1	1
Návod v češtině	ano	ano	ano	ano
Poměrné zastoupení sledovaných bakteriálních kmenů k celkové mase orálních mikroorganizmů	ne	ne	ne	ne
Detekce genů kódujících betalaktamázu (rezistence k antibiotikům)	ne	ne	ano	ano
Streptococcus mutans	ano	ano	ano	ne
Streptococcus sorbinus	ne	ne	ne	ne
***Lactobacillus* sp.**	ne	ano	ano	ne
***Actinomyces* sp. strain B19SC**	ne	ne	ano	ne
Aggregatibacter actinomycetemcomitans	ne	ne	ano	ano
Porphyromonas gingivalis	ne	ne	ano	ano
Tannerella forsythia	ne	ne	ano	ano
Treponema denticola	ne	ne	ano	ano
***Fusobacterium nucleatum* sp.**	ne	ne	ano	ano
Prevotella intermedia	ne	ne	ano	ano
Parvimonas micra	ne	ne	ano	ano
Výhody	• názornost • rychlost stanovení	• názornost	• citlivost kvalitativního stanovení	• citlivost kvalitativního stanovení
Nevýhody	• semikvantitativní stanovení • stanovení pouze *S. mutans* • bez stanovení citlivosti k antibiotikům	• pouze kvalitativní stanovení • bez stanovení citlivosti k antibiotikům	• semikvantitativní stanovení • cena	• semikvantitativní stanovení

Od firmy Ivoclar Vivadent lze zakoupit kombinovaný test CRT Intro Pack (1879 Kč), který obsahuje 3 testy CRT buffer Refill 6 a 3 testy CRT® bacteria Refill 6.

Firma upřednostňuje stanovení většího množství vzorků.

VariOr® Dento 11 sadou (1800 Kč) je možné navíc stanovit bakterie: *Campylobacter rectus*, *Eikenella corrodens*, *Eubacterium nodatum*, *Capnocytophaga* sp. (*C. ochracea*, *sputigena*, *gingivalis*).

VariOr® Caries	micro-IDent® ####	OralBac	PerioBac	CarioBac
GenTrend	Hain Lifescience GmbH (distributor v ČR Eurotex s.r.o.)	PřF MU	PřF MU	PřF MU
ne	ne	ne	ne	ne
ano	ano*	ano	ano	ano
do 5 pracovních dnů	do 2 týdnů	do 5 pracovních dnů	do 5 pracovních dnů	do 5 pracovních dnů
zubní plak	sulkulární tekutina	zubní plak + sulkulární tekutina	sulkulární tekutina	zubní plak
PCR	PCR	PCR	PCR	PCR
480 Kč + současně poslaný vzorek dospělého se vzorkem dítěte do 7 let zdarma + zdarma odběrový set	1767 Kč + zdarma odběrový set	1500 Kč + zdarma odběrový set	1200 Kč + zdarma odběrový set	450 Kč + zdarma odběrový set
1+1	1	1	1	1
ano	ano	ano	ano	ano
ne	ne	ano	ano	ano
ne	ne	ne	ne	ne
ano	ne	ano	ne	ano
ano	ne	ne	ne	ne
ne	ne	ano	ne	ano
ne	ne	ano	ne	ano
ne	ano	ano	ano	ne
ne	ano	ano	ano	ne
ne	ano	ano	ano	ne
ne	ano	ano	ano	ne
ne	ne	ano	ano	ne
ne	ano	ano	ano	ne
ne	ne	ano	ano	ne
• citlivost kvalitativního stanovení • stanovení vzorku dítěte k dospělému zdarma	• citlivost kvalitativního stanovení	• citlivost kvalitativního stanovení • kvantitativní stanovení • poměr k celkové mase orálních mikroorganizmů	• citlivost kvalitativního stanovení • kvantitativní stanovení • poměr k celkové mase orálních mikroorganizmů	• citlivost kvalitativního stanovení • kvantitativní stanovení • poměr k celkové mase orálních mikroorganizmů
• semikvantitativní stanovení	• semikvantitativní stanovení • bez stanovení citlivosti k antibiotikům	• bez stanovení citlivosti k antibiotikům	• bez stanovení citlivosti k antibiotikům	• bez stanovení citlivosti k antibiotikům

Sadou micro-IDent®plus (2481 Kč) je možné navíc stanovit bakterie: *P. micra*, *F. nucleatum/periodonticum*, *Campylobacter rectus*, *Eubacterium nodatum*, *Eikenella corrodens*, *Capnocytophaga species*

* Vzorky se standardně posílají do externí laboratoře, ale je zde možnost zakoupit si sady micro-IDent® (10 455 Kč/12 testů) a micro-IDent®plus (20 921 Kč/12 testů) i samostatně a stanovit si orální bakterie ve vlastní laboratoři s odpovídajícím laboratorním vybavením.

KOMERČNÍ TESTY PRO STANOVENÍ VROZENÉ NÁCHYLNOSTI K ONEMOCNĚNÍM DUTINY ÚSTNÍ

Testování vrozené náchylnosti k onemocnění závěsného aparátu zubu je v současnosti na evropském trhu nabízeno formou analýzy *IL-1* genu (případně genu kódujícího specifický sérotyp hlavního histokompatibilního systému HLA-DR4), v USA je komerčně dostupný i test na další genetický marker (IL-6 MyPerioID®, OralDNA® Labs, USA). Stanovované markery mají dle literárních údajů vztah i k periimplantitidě (24) a k řadě dalších systémových onemocnění, jako je diabetes mellitus, revmatická artritida či astma (25, 26, 27).

Na českém trhu jsou k dispozici test VariOr® Gen od firmy GenTrend, kterým je možné stanovit dědičný předpoklad k parodontitidě na základě analýzy dvou polymorfizmů v genech pro IL-1alfa (*IL-1A*) a IL-1beta (*IL-1B*) a variability v *HLA-DR4* genu, a test pro analýzu 3 polymorfizmů v *IL-1* genu GenoType® IL-1 (HAIN LIFESCIENCE GmbH) dostupný přes firmu Eurotex, s. r. o., viz **tab. III**. Oba testy jsou primárně připraveny jako doplněk testů VariOr® Dento 11 nebo micro-IDent®, ovšem test pro zjištění vrozené náchylnosti k zánětlivým onemocněním je možné provést i samostatně.

Postup je velmi jednoduchý, lékař si objedná odběrovou sadu, dle instrukcí provede odběr vzorku buněk z bukální sliznice (lze použít i papírový čep se sulkulární tekutinou, která byla odebrána pro stanovení parodontálních bakterií) a odešle s žádankou vzorek do laboratoře k analýze. Principiálně je test opět založen na PCR a výsledky je možné získat do 5 až 14 dní od zaslání do laboratoře. Vzhledem k tomu, že **většina parodontitid nepatří k tzv. monogenním chorobám, nelze toto testování zatím jednoznačně doporučit k hodnocení zvýšeného rizika k rozvoji onemocnění v klinické praxi**.

Zatím není nabízen žádný test pro stanovení individuálního rizika vzniku a rozvoje zubního kazu, jsou však snahy o identifikaci genových variant definujících fenotyp mnohočetného kazu v dočasné i stálé dentici (28).

Tabulka III: *Přehled komerčních testů pro stanovení genetické predispozice k onemocnění parodontálních tkání – dostupných v ČR*

Název kitu	**VariOr® Gen #**	**GenoType® IL-1**
Firma	GenTrend	Hain Lifescience GmbH (distributor v ČR Eurotex, s. r. o.)
Možnost provést vyhodnocení přímo v ordinaci	ne	ne
Nutnost přístrojového vybavení	ano	ano*
Rychlost stanovení	do 5 pracovních dnů	do 2 týdnů
Vstupní vzorek	stěr z bukální sliznice	stěr z bukální sliznice
Princip stanovení	PCR	PCR
Cena s DPH	1500 Kč + zdarma odběrový set	1767 Kč + zdarma odběrový set
Návod v češtině	ano	ano
Co zjistíme	• *IL-1A* genotyp • *IL-1B* genotyp • HLA-DR4 sérotyp	• *IL-1A* genotyp • *IL-1B* genotyp • *IL-1RN* genotyp
Výhody	• **rychlejší dodání výsledků**	

\# Analýzu VariOr® Gen lze provést ze vzorku pro VariOr® Dento 11, pacient tedy není zatěžován dalším odběrem. Pro kombinaci testů platí zvýhodněná cena 2500 Kč.

* Vzorky se standardně posílají do externí laboratoře, ale je zde možnost zakoupit si sadu GenoType® IL-1 (12 testů) i samostatně a stanovit si orální bakterie ve vlastní laboratoři s odpovídajícím laboratorním vybavením.

DALŠÍ KOMERČNÍ TESTY

Jako nástroj při prevenci onemocnění tvrdých i měkkých zubních tkání nebo při detekci zubního kazu je možné využívat také komerčně dostupné roztoky, výplachy, gely nebo tablety k vizualizaci zubního plaku nebo pro označení kariézních defektů, kazů v dentinu, případně i vstupů do kořenových kanálků. Tyto produkty však obsahují barviva (např. erythrosin, briliantová modř, patentní modř V, fluorescenční barviva), která mohou, dle některých literárních údajů, představovat zdravotní riziko (29, 30), zvláště pokud by byly aplikovány u dětí. Specifikace těchto produktů jsme do naší přehledové práce nezačlenili.

ZÁVĚR

S vyšší životní úrovní západní civilizace roste poptávka pacientů po **nadstandardní zdravotní péči**, ať už se jedná o přímé výkony, nebo i o laboratorní testování. Na českém trhu (eventuálně v EU) jsou nabízeny testy, kterými je možné zjišťovat predispozice pacienta ke vzniku zubního kazu a onemocnění parodontu na základě analýzy jejich sliny, bakterií v dutině ústní a IL-1 genového polymorfizmu. Komerčně dostupné testy mohou být jednotlivé i kombinované. Při analýze sliny jsou testy navrženy tak, aby bylo možné stanovení obsahu vody a hydratace sliny i jejího průtoku, a/nebo pufrovací kapacity sliny a pH. Orální mikroflóra je testována v zubním plaku, ve slinách i v sulkulární tekutině. Sleduje se její složení, měří se počet specifických bakterií (případně i jejich aktivita), jejich procentuální zastoupení, i rezistence k některým typům antibiotik.

Názorná prezentace výsledků analýz vlastností sliny a do jisté míry snad i orální mikroflóry může být vhodným intervenčním nástrojem praktického zubního lékaře **k motivaci pacienta k péči o jeho orální zdraví**. Vzhledem ke komplexnímu charakteru vybraných orálních onemocnění je vždy nutné výsledky získané laboratorními testy posuzovat společně s detailním klinickým nálezem.

Poznámka: Na českém trhu bylo v minulých letech možné zakoupit testy od firmy Orion Diagnostika (Dentobuff® strip, Dentocult® SM Strip mutans, Dentocult® LB, Dentocult® CA), dle vyjádření zástupce firmy pro ČR se zmíněné testy již od roku 2017 nevyrábějí. Obdobně již není možné zakoupit test Cario-Gene® od firmy Protean. Všechny ceny testů jsou uvedeny s DPH k datu 14. 2. 2018 a bez poštovného.

Poděkování

Článek vznikl s podporou Ministerstva zdravotnictví České republiky, grant č. NV 17-30439A, grantu GAČR GB14-37368G, projektu Specifický výzkum MUNI/A/1008/2017 a z prostředků poskytnutých Lékařskou fakultou MU juniorskému výzkumníkovi Petře Bořilové Linhartové.

Konflikt zájmů

Autoři nemají žádný konflikt zájmů, vazbu na osoby zabývající se výrobou či distribucí uvedených produktů máme jen v rovině výzkumné, případně jako běžní zákazníci.

Literatura

1. **Jepsen S, Blanco J, Buchalla W, et al.** Prevention and control of dental caries and periodontal diseases at individual and population level: consensus report of group 3 of joint EFP/ORCA workshop on the boundaries between caries and periodontal diseases. J Clin Periodontol, 2017, 44 (Suppl 18): S85–S93. doi: 10.1111/jcpe.12687.
2. **Bořilová Linhartová P, Izakovičová Hollá L.** Léčiva a lékové formy jako rizikové faktory pro zubní kaz. Česká a slovenská farmacie, 2017, 66(3): 103–106.
3. **Bradshaw DJ, Lynch RJ.** Diet and the microbial aetiology of dental caries: new paradigms. Int Dent J, 2013 63 (Suppl 2): 64–72. doi: 10.1111/idj.12082.
4. **Aas JA, Griffen AL, Dardis SR, et al.** Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol, 2008, 46(4): 1407–17. doi: 10.1128/JCM.01410-07.
5. **Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr.** Microbial complexes in subgingival plaque. J Clin Periodontol, 1998, 25(2): 134–144. doi:10.1111/j.1600 – 051X.1998.tb02419.x.
6. **Tettamanti L, Gaudio RM, Cura F, Mucchi D, Illuzzi N, Tagliabue A.** Prevalence of periodontal pathogens among italian patients with chronic periodontitis: a retrospective study on 2992 patients. Oral Implantol (Rome), 2017, 10(1): 28–36. doi: 10.11138/orl/2017.10.1.028.
7. **Van der Velden U.** What exactly distinguishes aggressive from chronic periodontitis: is it mainly a difference in the degree of bacterial invasiveness? Periodontol 2000, 2017, 75(1): 24–44. doi: 10.1111/prd.12202.
8. **Könönen E, Müller HP.** Microbiology of aggressive periodontitis. Periodontol 2000, 2014, 65(1): 46–78. doi: 10.1111/prd.12016.
9. **Nibali L.** Aggressive Periodontitis: microbes and host response, who to blame? Virulence, 2015, 6(3): 223–228. doi: 10.4161/21505594.2014.986407.
10. **Mombelli A.** Microbial colonization of the periodontal pocket and its significance for periodontal therapy. Periodontol 2000, 2018, 76(1): 85–96. doi: 10.1111/prd.12147.
11. **Lai PC, Schibler MR, Walters JD**. Azithromycin enhances phagocytic killing of Aggregatibacter actinomycetemcomitans Y4 by human neutrophils. J Periodontol, 2015, 86(1): 155–161. doi: 10.1902/jop.2014.140183.
12. **Huth KC, Quirling M, Lenzke S, Paschos E, et al.** Effectiveness of ozone against periodontal pathogenic microorganisms. Eur J Oral Sci, 2011, 119(3): 204–210. doi: 10.1111/j.1600-0722.2011.00825.x.
13. **Fernandes T, Bhavsar C, Sawarkar S, D'souza A.** Current and novel approaches for control of dental biofilm. Int J Pharm, 2018, 536(1): 199–210. doi: 10.1016/j.ijpharm.2017.11.019.
14. **da Silva MK, de Carvalho ACG, Alves EHP, da Silva FRP, Pessoa LDS, Vasconcelos DFP.** Genetic Factors and the Risk of Periodontitis Development: Findings from a Systematic Review Composed of 13 Studies of Meta-Analysis with 71,531 Participants. Int J Dent, 2017, 2017: 1914073. doi: 10.1155/2017/1914073.
15. **Bořilová Linhartová P, Valová S, Kaštovský J, et al.** Genová variabilita v imunoregulačních faktorech u pacientů s chronickou parodontitidou a diabetes mellitus. Čes. stomatol. Prakt. zubní lék., 2015, 115 (4), 89–97.
16. **Ramsay DS, Rothen M, Scott JM, Cunha-Cruz J.** Tooth wear and the role of salivary measures in general practice patients. Clin Oral Investig, 2015, 19(1): 85–95. doi: 10.1007/s00784-014-1223-4.
17. **Maldupa I, Brinkmane A, Mihailova A.** Comparative analysis of CRT Buffer, GC saliva check buffer tests and laboratory titration to evaluate saliva buffering capacity. Stomatologija, 2011, 13(2): 55–61.
18. **Cheaib Z, Ganss C, Lamanda A, Turgut MD, Lussi A.** Comparison of three strip-type tests and two laboratory methods for salivary buffering analysis. Odontology, 2012, 100(1): 67–75. doi: 10.1007/s10266-011-0018-1.
19. **Maheaswari R, Kshirsagar JT, Lavanya N.** Polymerase chain reaction: A molecular diagnostic tool in periodontology. J Indian Soc Periodontol, 2016, 20(2): 128–135. doi: 10.4103/0972-124X.176391.
20. **Poskerová H, Bořilová Linhartová P, Cvanová M, Izakovičová Hollá L.** Vliv onemocnění diabetes mellitus na stav chrupu a parodontu. Čes. stomatol. Prakt. zubní lék., 2018, 118(1): 3–12.
21. **Borilova Linhartova P, Vokurka J, Poskerova H, Fassmann A, Izakovicova Holla L.** Haplotype analysis of interleukin-8 gene polymorphisms in chronic and aggressive periodontitis. Mediators Inflamm, 2013, 2013: 342351.
22. **Borilova Linhartova P, Kastovsky J, Lucanova S, et al.** Interleukin-17A gene variability in patients with type 1 diabetes mellitus and chronic periodontitis: its correlation with IL-17 levels and the occurrence of periodontopathic bacteria. Mediators Inflamm, 2016, 2016: 2979846.
23. **Hughes D, Andersson DI.** Environmental and genetic modulation of the phenotypic expression of antibiotic resistance. FEMS Microbiol Rev, 2017, 41(3): 374–391. doi: 10.1093/femsre/fux004.
24. **Liao J, Li C, Wang Y, et al.** Meta-analysis of the association between common interleukin-1 polymorphisms and dental implant failure. Mol Biol Rep, 2014, 41(5): 2789–2798. doi: 10.1007/s11033-014-3133-6.
25. **Luotola K, Pietilä A, Zeller T, et al.** Associations between interleukin-1 (IL-1) gene variations or IL-1 receptor antagonist levels and the development of type 2 diabetes. J Intern Med, 2011, 269(3): 322–332. doi: 10.1111/j.1365-2796.2010.02294.x.
26. **Lee YH, Bae SC.** Associations between interleukin-1 and IL-1 receptor antagonist polymorphisms and susceptibility to rheumatoid arthritis: A meta-analysis. Cell Mol Biol (Noisy-le-grand), 2015, 61(8): 105–111.
27. **He Y, Peng S, Xiong W, Xu Y, Liu J.** Association between polymorphism of interleukin-1 beta and interleukin-1 receptor antagonist gene and asthma risk: a meta-analysis. Scientific World Journal, 2015, 2015: 685684. doi: 10.1155/2015/685684.
28. **Kornman KS, Polverini PJ.** Clinical application of genetics to guide prevention and treatment of oral diseases. Clin Genet, 2014, 86(1): 44–49. doi: 10.1111/cge.12396.
29. **Tanaka T.** Reproductive and neurobehavioural toxicity study of erythrosine administered to mice in the diet. Food Chem Toxicol, 2001, 39(5): 447–454. doi: 10.1016/S0278-6915(00)00163-0.
30. **Yuan CJ, Marikawa Y.** Developmental toxicity assessment of common excipients using a stem cell-based in vitro morphogenesis model. Food Chem Toxicol, 2017, 109(Pt 1): 376–385. doi: 10.1016/j.fct.2017.09.023.

Attachment 8 Article "Lack of association between *BMP2/DLX3* gene polymorphisms and dental caries in primary and permanent dentitions"

Short Communication

Caries Research

Caries Res 2017;51:590–595
DOI: 10.1159/000479828

Received: June 19, 2017
Accepted after revision: July 27, 2017
Published online: October 24, 2017

Lack of Association between *BMP2/DLX3* Gene Polymorphisms and Dental Caries in Primary and Permanent Dentitions

Jakub Kastovsky[a, b] Petra Borilova Linhartova[a, b]
Kristina Musilova[a] Lenka Zackova[c] Martina Kukletova[a]
Lubomir Kukla[d] Lydie Izakovicova Holla[a, b, d]

[a]Clinic of Stomatology, Institution Shared with St. Anne's Faculty Hospital, [b]Department of Pathophysiology, and [c]Stomatology Research Center, Faculty of Medicine, and [d]Research Centre for Toxic Compounds in the Environment (RECETOX), Chemistry Section, Faculty of Science, Masaryk University, Brno, Czech Republic

Keywords
BMP2 · Case-control study · Dental caries · DLX3 · Gene polymorphism

Abstract
The aim of this study was to analyze the association between *BMP2* (rs1884302) and *DLX3* (rs2278163) gene polymorphisms and dental caries in primary and permanent dentitions. A total of 914 subjects were genotyped by the TaqMan methods: 176 caries-free children (with Decayed/Missing/Filled Teeth, DMFT = 0), 542 patients with dental caries in permanent dentition (DMFT ≥1), 83 caries-free children with primary teeth (with decayed/missing/filled teeth, dmft = 0), and 113 children with early childhood caries (ECC, dmft ≥1). There were no significant differences in allele/genotype frequencies between patients with caries in permanent dentition/ECC and caries-free children or between patients with very low (DMFT = 0–2), low (DMFT = 3–5), moderate (DMFT = 6–8), or high (DMFT ≥9) caries experience. Variability in *BMP2* and *DLX3* was not associated with caries in the Czech population.
© 2017 S. Karger AG, Basel

Dental caries is a multifactorial disease involving a complex interplay between heredity and environmental factors. It is associated with poor oral hygiene, high occurrence of certain bacteria (e.g., *Streptococcus mutans*) in the oral cavity, low salivary flow and saliva composition, insufficient fluoride exposure, tooth morphology, and genetic predispositions, among others [Macek et al., 2003; Selwitz et al., 2007]. According to Wang et al. [2010], genetic contribution to dental caries is higher than 50%. Furthermore, genetic variations may also impact enamel development and consequently individual caries susceptibility, and these effects may be distinct between primary and permanent dentition [Bayram et al., 2015]. In the recent genome-wide association study, a polymorphism in one of the bone morphogenetic proteins (*BMP*), a tooth development gene, was found to be significantly associated with dental caries susceptibility [Morrison et al., 2016].

J.K. and P.B.L. contributed equally to this study.

KARGER © 2017 S. Karger AG, Basel

E-Mail karger@karger.com
www.karger.com/cre

Prof. Lydie Izakovicova Holla, MD, PhD
Clinic of Stomatology and Department of Pathophysiology
Faculty of Medicine, Masaryk University
Pekarska 664/53, CZ–602 00 Brno (Czech Republic)
E-Mail holla@med.muni.cz

Downloaded by:
Masaryk University
147.251.6.77 - 10/24/2017 10:17:05 AM

BMPs are a group of signaling molecules which belong to the transforming growth factor-β superfamily of proteins. The BMP2 signaling pathway is an essential regulator for bone tissue formation and is required for the initiation of fracture healing [Wu et al., 2016]. BMP2-induced differentiation of osteoblast cells via osteoactin transcription is differentially regulated by distal-less homeobox 3 (DLX3), DLX5, and MSX2 during osteoblast differentiation [Singh et al., 2012]. BMP2 has been reported to stimulate the expression of dentin sialophosphoprotein (the main component of dentin matrix) [Iohara et al., 2006]; production of this protein is strongly reduced in the absence of DLX3 in odontoblasts [Duverger et al., 2012]. Moreover, Viale-Bouroncle et al. [2012] in their study showed that DLX3 supported the osteogenic differentiation in the dental follicle cells via a BMP2 positive feedback loop, and the presence of DLX3 protein is considered a critical factor in the development of teeth.

Ohta et al. [2015], who analyzed 7 single nucleotide polymorphisms (SNPs) in the gene for DLX3 in a total of 201 Japanese children aged 5–6 years with primary teeth, found a significant association between the *DLX3* gene polymorphism (rs2278163) and caries. In addition, Romanos et al. [2015] reported that the *BMP2* gene polymorphism (rs1884302) was associated with caries experience only in the primary dentition. However, the analysis of the *BMP2* and *DLX3* polymorphisms with caries according to dmft/DMFT (decayed/missing/filled teeth/Decayed/Missing/Filled Teeth) indexes in primary and also permanent dentitions should be performed, as Wang et al. [2010] suggested that the genes affecting susceptibility to caries in the primary dentition may differ from those in the permanent teeth. We focused on the analysis of 2 previously associated SNPs in the *BMP2* (in Brazilians) and *DLX3* (in Japanese population) genes and dental caries susceptibility in Caucasians (Czech children). The variants *BMP2* (rs1884302) and *DLX3* (rs2278163) were examined in the groups of 13- to 15-year-old children with permanent dentition and 2- to 6-year-old children with early childhood caries (ECC) in the primary teeth; their possible association with susceptibility to or severity of caries was tested.

Methods

Subjects

The total of 718 unrelated Caucasian children (378 boys and 340 girls), aged 13–15 years, selected from the European Longitudinal Study of Pregnancy and Childhood (ELSPAC) Brno study comprising over 5,738 children and their families, were investigated [Piler et al., 2016]. The children underwent dental examination at the Clinic of Stomatology at St. Anne's University Hospital and the Faculty of Medicine, Masaryk University, as described previously [Volckova et al., 2014; Izakovicova Holla et al., 2015]. The study group comprised caries-affected subjects (with DMFT ≥1; n = 542; 285 boys and 257 girls) and caries-free children (with DMFT = 0; n = 176; 93 boys and 83 girls). The definition of caries experience level based on age and DMFT was used according to Vieira et al. [2008].

A convenience sample of 113 preschool Czech children (64 boys and 49 girls) with ECC (dmft ≥1), aged 2–6 years, who were referred to the Paediatric Section of the Department of Dentistry, St. Anne's Faculty Hospital, and the Faculty of Medicine, Masaryk University, in Brno for complex treatment under general anesthesia, was recruited; the studied group has been described previously [Borilova Linhartova et al., 2016]. In addition, 83 young children of Czech origin with primary teeth (39 boys and 44 girls) without dental caries (dmft = 0), aged 3–6 years, were selected as a control group.

The inclusion criteria consisted of simple informed consent of the children and their parents and expression of their willingness to participate. The study was approved by the Committee for Ethics of the Faculty of Medicine, Masaryk University, Brno (3/2004, from March 30, 2004) and St. Anne's Faculty Hospital (without number, from April 13, 2004, and 1G/2017). The informed consent was obtained from all legal guardians of the children, in line with the Helsinki Declaration, prior to their inclusion in the study.

SNP Genotyping TaqMan® Assay

Isolation and storage of DNA samples were conducted in the laboratory of the Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic [Borilova Linhartova et al., 2016].

Genotyping of SNPs in *BMP2* (rs1884302) and *DLX3* (rs2278163) was based on polymerase chain reaction using 5′ nuclease TaqMan® assays (C_11673995_10 and C_2902913_20, respectively). Reaction mixture and conditions were designed according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA), and fluorescence was measured using the ABI PRISM 7000 Sequence Detection System. SDS version 1.2.3 software was used to analyze real-time and endpoint fluorescence data. Genotyping was performed by investigators J.K. and P.B.L., who were unaware of the phenotype, and 10% of the samples were analyzed in duplicate.

Statistical Analysis

Standard descriptive statistical methods were applied in the analysis. The allele frequencies were calculated from the observed numbers of the genotypes. The differences in the allele frequencies were counted using the Fisher exact test; the χ^2 test was used for comparison of the genotype frequencies. The same analysis was also used to test for deviation of the genotype distribution from Hardy-Weinberg equilibrium. Only values $p < 0.05$ were considered statistically significant. Logistic regression analysis was implemented using risk factors (age, gender), with *BMP2* or *DLX3* SNPs as covariates in order to test gene-environment interactions. Statistical analyses were computed using the software packages Statistica v. 12 (StatSoft Inc., Tulsa, OK, USA). Power analysis was performed with respect to a case-control design of the study and was done using software PASS 13 [Hintze, 2014].

Downloaded by:
Masaryk University
147.251.5.77 - 10/24/2017 10:17:05 AM

Table 1. The distribution of the *BMP2* and *DLX3* allele and genotype frequencies in children with permanent or primary dentition according to their caries experience

	Total	*BMP2* (rs1884302)							*DLX3* (rs2278163)						
		TT	TC	CC	*p* value	T	C	*p* value	GG	GA	AA	*p* value	G	A	*p* value
Children with permanent dentition															
DMFT = 0	176	80 (45.5)	75 (42.6)	21 (11.9)		235 (66.8)	117 (33.2)		113 (64.2)	53 (30.1)	10 (5.7)		279 (79.3)	73 (20.7)	
DMFT ≥1	542	259 (47.8)	234 (43.2)	49 (9.0)	0.52	752 (69.4)	332 (30.6)	0.20	323 (59.6)	197 (36.3)	22 (4.1)	0.26	843 (77.8)	241 (22.2)	0.31
DMFT = 0–2	388	183 (47.2)	165 (42.5)	40 (10.3)		531 (68.4)	245 (31.6)		241 (62.1)	129 (33.2)	18 (4.6)		611 (78.7)	165 (21.3)	
DMFT = 3–5	204	94 (46.1)	95 (46.6)	15 (7.4)	0.41	283 (69.4)	125 (30.6)	0.40	126 (61.8)	70 (34.3)	8 (3.9)	0.90	322 (78.9)	86 (21.1)	0.50
DMFT = 6–8	80	39 (48.7)	29 (36.3)	12 (15.0)	0.37	107 (66.9)	53 (33.1)	0.38	42 (52.5)	34 (42.5)	4 (5.0)	0.26	118 (73.8)	42 (26.2)	0.10
DMFT ≥9	46	23 (50.0)	20 (43.5)	3 (6.5)	0.71	66 (71.7)	26 (28.3)	0.30	27 (58.7)	17 (37.0)	2 (4.3)	0.88	71 (77.2)	21 (22.8)	0.41
Children with primary dentition															
dmft = 0	83	33 (39.8)	39 (47.0)	11 (13.2)		105 (63.3)	61 (36.7)		48 (57.8)	32 (38.6)	3 (3.6)		128 (77.1)	38 (22.9)	
dmft ≥1 (ECC)	113	43 (38.1)	58 (51.3)	12 (10.6)	0.78	144 (63.7)	82 (36.3)	0.50	70 (61.9)	36 (31.9)	7 (6.2)	0.50	176 (77.9)	50 (22.1)	0.48

Values represent numbers (%) of subjects. dmft/DMFT, decayed/missing/filled teeth/Decayed/Missing/Filled Teeth; ECC, early childhood caries.

Results

The observed *BMP2* (rs1884302) and *DLX3* (rs2278163) genotype distributions in the control group and in children with caries experience were in Hardy-Weinberg equilibrium ($p > 0.05$). With the standard statistical procedures applied (power 80%, alpha error 5%), the recruited sample size was sufficient to detect significant differences ≥10% in the relative rate of *BMP2* and *DLX3* markers between the subgroups.

There were no significant differences in the allele or genotype frequencies between the caries-free children (DMFT = 0) and patients with DMFT ≥1 ($p > 0.05$), or between the children with very low (DMFT = 0–2), low (DMFT = 3–5), moderate (DMFT = 6–8), or high (DMFT ≥9) caries experience ($p > 0.05$). Similarly, no significant associations between these 2 gene variants and caries in young children with ECC were found ($p > 0.05$) (see Table 1). Analysis according to caries experience levels [Vieira et al., 2008] could not be performed in children with ECC as all of these subjects had dmft ≥8 (range 8–20, mean ± SD 13.2 ± 2.4).

The logistic regression showed a lack of *BMP2*/*DLX3* gene-by-sex and gene-by-age interactions in relation to the caries phenotype ($p > 0.05$); therefore, the sex- and age-stratified univariate analyses were not performed (data not shown).

Discussion

Cariogenic bacteria are part of dental plaque which accumulates on the hard tissues in the oral cavity. The alterations in tooth morphology can lead to the increased establishment of this complex biofilm and thus to dental caries. Some studies have focused on genetic variants in genes expressed in enamel development [Deeley et al., 2008; Patir et al., 2008; Kang et al., 2011; Olszowski et al., 2012; Shimizu et al., 2012; Antunes et al., 2014; Ohta et al., 2015; Romanos et al., 2015]. BMP2 signaling guides the osteogenic differentiation in dental follicle cells by induction of the transcription factor DLX3, thus the genes encoding these proteins can be identified as "candidates" for dental caries. Recently, a family-based association study by Jeremias et al. [2016] suggested that variations in genes implicated in amelogenesis, including *DLX3* and *BMP2*, were associated with the susceptibility to develop molar-incisor hypomineralization.

BMPs play an important role during the initial process of enamel formation and regulate cartilage develop-

Downloaded by:
Masaryk University
147.251.6.77 - 10/24/2017 10:17:05 AM

ment [Wang et al., 2014]. Romanos et al. [2015] found an association between the *BMP2* gene polymorphism (rs1884302) and caries experience in the primary teeth of a Brazilian population after multivariate regression analysis, but to date no study dealing with the *BMP2* gene polymorphism and dental caries in a Caucasian population has been published. The presented results are in disagreement with the findings of the Brazilian authors as no significant differences between children without/with dental caries in the primary or permanent dentition were found in the Czech population. In line with the minor allele frequency (MAF = 0.33, C allele) of *BMP2* SNP (rs1884302) for the Caucasian population according to the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=1884302), the representation of this allele is independent of age and dental caries score in 32% of subjects (Table 1). However, MAF even 10% higher was found in a Brazilian population. In addition, the authors did not find any associations between the genotype and allele distribution for the *BMPs* polymorphisms and primary enamel microhardness alterations [Romanos et al., 2015].

Further, 2 genetic disorders caused by defects in the *DLX3* gene are known to exist. The first one is an autosomal dominant disease caused by the deletion of 4 base pairs – tricho-dento-osseous syndrome [Duverger et al., 2013] – which is manifested by defects in the structure of hair, teeth, and problems associated with bone development. Zhang et al. [2015] suggested that DLX3 promoted the expression of the enamel matrix protein genes in amelogenesis, while mutant DLX3 disrupted this regulatory function. The deletion of 2 base pairs in homeobox causes the hypomaturation hypoplastic form of amelogenesis imperfecta with taurodontism, which ends by the emergence of anomalies in the shape of crowns and roots of molars [Dong et al., 2005; Kim et al., 2016]. The murine chromosome 11 appeared to be positive for susceptibility to dental caries [Ohta, 2005], and the *DLX* gene is located on this chromosome. A few studies suggested the association between amelogenesis imperfecta and dental caries; mechanical tests showed that dental enamel with less amelogenin was "weaker," while the dental enamel of animals over-expressing amelogenin appeared to be more resistant to acid dissolution [Vieira et al., 2015].

Ohta et al. [2015] found the association between the T allele of *DLX3* polymorphism (rs2278163) and the development of caries, but only in patients with a higher frequency of *S. mutans* and *S. sobrinus* (more than 10,000 CFU/mL); this allele was more common in patients with dmft ≥3 than in those with dmft ≤2 in the primary dentition ($p < 0.014$). Nevertheless, there were no differences in the allele and genotype frequencies in *DLX3* SNP (rs2278163) between caries-free children and children with caries in permanent dentition/ECC, and even no association was found with the severity of caries in the permanent dentition in our study. In the NCBI database, MAF (A allele) of *DLX3* SNP (rs2278163) is 0.24 for Caucasians (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2278163). Similarly, 22% of A allele carriers, independently of dental caries score, were found in our cohort, while the MAF (C allele) in 198 Japanese children was only 31% [Ohta et al., 2015]. These interpopulation differences in representation of the alleles of both SNPs must be taken into account when comparing the results of studies by Romanos et al. [2015] and Ohta et al. [2015] with our study.

There are some potential limitations to the present study that need to be considered. Firstly, case-control design carries the risk of providing false-positive results, but our cohort consisted of 914 subjects, so the risk of a false-negative conclusion was relatively low. Although Romanos et al. [2015] investigated a larger population (1,731 subjects), their cohort was mixed (Brazilian Caucasians and Afro-descendants), and significant results were obtained only after a multivariate regression analysis. Ohta et al. [2015] examined 198 Japanese children with primary dentition, but no study investigated a relation between *DLX3* SNPs and dental caries in permanent dentition. Thus, this is the first study that analyzed the *BMP2* and *DLX3* polymorphisms in relation to dmft/DMFT scores in a European Caucasian population. Secondly, neither the enamel microhardness analysis nor the detection of cariogenic bacteria in the oral cavity were performed in this study. Thirdly, we did not obtain bite-wing radiographs for ethical reasons to avoid radiation exposure of young children; thus, small interproximal lesions may not have been detected. Finally, we focused just on 2 previously associated polymorphisms in the *BMP2* and *DLX3* genes and did not investigate other candidate genes or polymorphisms. In multifactorial complex diseases, such as dental caries, each genetic polymorphism has usually only a small effect, and interactions among gene variants and other environmental factors can potentially affect the overall phenotype.

In conclusion, variability in the *BMP2* and *DLX3* genes was not associated with dental caries in primary and permanent dentition in Czech children. Future

Downloaded by:
Masaryk University
147.251.6.77 - 10/24/2017 10:17:05 AM

studies are necessary for understanding the role of the candidate enamel formation genes in dental caries susceptibility.

Acknowledgments

The study was supported by funds from the Faculty of Medicine, Masaryk University, to junior researcher Petra Borilova Linhartova, by project MUNI/A/0948/2016, and by grants AZV 17-30439A, GACR GB14-37368G, and Recetox RI (LM2015051 and CZ.02.1.01/0.0/0.0/16_013/0001761).

Disclosure Statement

The authors declare no conflict of interest.

Author Contributions

P.B.L., J.K., M.K., L.K., and L.I.H. designed the study and drafted the paper. K.M., L.Z., and M.K. performed the clinical analyses and collected blood/saliva samples. J.K. and P.B.L. carried out the molecular analysis, and L.I.H. performed the statistical analysis. All authors revised the final version of the manuscript.

References

Antunes LS, Tannure PN, Antunes LA, Reis MF, Costa MC, Gouvêa CV, Olej B, Granjeiro JM, Küchler EC: Genetic association for caries susceptibility among cleft lip and/or palate individuals. J Contemp Dent Pract 2014;15:288–293.

Bayram M, Deeley K, Reis MF, Trombetta VM, Ruff TD, Sencak RC, Hummel M, Dizak PM, Washam K, Romanos HF, Lips A, Alves G, Costa MC, Granjeiro JM, Antunes LS, Küchler EC, Seymen F, Vieira AR: Genetic influences on dental enamel that impact caries differ between the primary and permanent dentitions. Eur J Oral Sci 2015, DOI: 10.1111/eos.12204.

Borilova Linhartova P, Kastovsky J, Bartosova M, Musilova K, Zackova L, Kukletova M, Kukla L, Izakovicova Holla L: ACE insertion/deletion polymorphism associated with caries in permanent but not primary dentition in Czech children. Caries Res 2016;50:89–96.

Deeley K, Letra A, Rose EK, Brandon CA, Resick JM, Marazita ML, Vieira AR: Possible association of amelogenin to high caries experience in a Guatemalan-Mayan population. Caries Res 2008;42:8–13.

Dong J, Amor D, Aldred MJ, Gu T, Escamilla M, MacDougall M: DLX3 mutation associated with autosomal dominant amelogenesis imperfecta with taurodontism. Am J Med Genet A 2005;133A:138–141.

Duverger O, Zah A, Isaac J, Sun HW, Bartels AK, Lian JB, Berdal A, Hwang J, Morasso MI: Neural crest deletion of Dlx3 leads to major dentin defects through down-regulation of Dspp. J Biol Chem 2012;287:12230–12240.

Duverger O, Isaac J, Zah A, Hwang J, Berdal A, Lian JB, Morasso MI: In vivo impact of Dlx3 conditional inactivation in neural crest-derived craniofacial bones. J Cell Physiol 2013;228:654–664.

Hintze J: PASS 13. NCSS, LLC, Kaysville, UT, USA. 2014. www.ncss.com.

Iohara K, Zheng L, Ito M, Tomokiyo A, Matsushita K, Nakashima M: Side population cells isolated from porcine dental pulp tissue with self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis. Stem Cells 2006;24:2493–2503.

Izakovicova Holla L, Borilova Linhartova P, Lucanova S, Kastovsky J, Musilova K, Bartosova M, Kukletova M, Kukla L, Dusek L: *GLUT2* and *TAS1R2* polymorphisms and susceptibility to dental caries. Caries Res 2015;49:417–424.

Jeremias F, Pierri RA, Souza JF, Fragelli CM, Restrepo M, Finoti LS, Bussaneli DG, Cordeiro RC, Secolin R, Maurer-Morelli CV, Scarel-Caminaga RM,Santos-Pinto L: Family-based genetic association for molar-incisor hypomineralization. Caries Res 2016;50:310–318.

Kang SW, Yoon I, Lee HW, Cho J: Association between AMELX polymorphisms and dental caries in Koreans. Oral Dis 2011;17:399–406.

Kim YJ, Seymen F, Koruyucu M, Kasimoglu Y, Gencay K, Shin TJ, Hyun HK, Lee ZH, Kim JW: Unexpected identification of a recurrent mutation in the *DLX3* gene causing amelogenesis imperfecta. Oral Dis 2016;22:297–302.

Macek MD, Beltrán-Aguilar ED, Lockwood SA, Malvitz DM: Updated comparison of the caries susceptibility of various morphological types of permanent teeth. J Public Health Dent 2003;63:174–182.

Morrison J, Laurie CC, Marazita ML, Sanders AE, Offenbacher S, Salazar CR, Conomos MP, Thornton T, Jain D, Laurie CA, Kerr KF, Papanicolaou G, Taylor K, Kaste LM, Beck JD, Shaffer JR: Genome-wide association study of dental caries in the Hispanic Communities Health Study/Study of Latinos (HCHS/SOL). Hum Mol Genet 2016;25:807–816.

Ohta M: Identification of chromosome region(s) influencing initial dental caries susceptibility in mice. Ped Dent J 2005;15:79–84.

Ohta M, Nishimura H, Asada Y: Association of DLX3 gene polymorphism and dental caries susceptibility in Japanese children. Arch Oral Biol 2015;60:55–61.

Olszowski T, Adler G, Janiszewska-Olszowska J, Safranow K, Kaczmarczyk M: *MBL2*, *MASP2*, *AMELX*, and *ENAM* gene polymorphisms and dental caries in Polish children. Oral Dis 2012;18:389–395.

Patir A, Seymen F, Yildirim M, Deeley K, Cooper ME, Marazita ML, Vieira AR: Enamel formation genes are associated with high caries experience in Turkish children. Caries Res 2008;42:394–400.

Piler P, Kandrnal V, Kukla L, Andrýsková L, Švancara J, Jarkovský J, Dušek L, Pikhart H, Bobák M, Klánová J: Cohort Profile: The European Longitudinal Study of Pregnancy and Childhood (ELSPAC) in the Czech Republic. Int J Epidemiol 2016, DOI: 10.1093/ije/dyw091.

Romanos HF, Antunes LS, Lopes LB, Saboia Tde M, Tannure PN, Lips A, Antunes LA, Abreu FV, Deeley K, Alves G, Granjeiro JM, Vieira AR, Costa MC, Kuchler EC: *BMP2* is associated with caries experience in primary teeth. Caries Res 2015;49:425–433.

Selwitz RH, Ismail AI, Pitts NB: Dental caries. Lancet 2007;369:51–59.

Singh M, Del Carpio-Cano FE, Monroy MA, Popoff SN, Safadi FF: Homeodomain transcription factors regulate BMP-2-induced osteoactivin transcription in osteoblasts. J Cell Physiol 2012;227:390–399.

Shimizu T, Ho B, Deeley K, Briseno-Ruiz J, Faraco IM Jr, Schupack BI, Brancher JA, Pecharki GD, Kuchler EC, Tannure PN, Lips A, Vieira TC, Patir A, Yildirim M, Poletta FA, Mereb JC, Resick JM, Brandon CA, Orioli IM, Castilla EE, Marazita ML, Seymen F, Costa MC, Granjeiro JM, Trevilatto PC, Vieira AR: Enamel formation genes influence enamel microhardness before and after cariogenic challenge. PLoS One 2012;7:e45022.

Downloaded by:
Masaryk University
147.251.6.77 - 10/24/2017 10:17:05 AM

Viale-Bouroncle S, Felthaus O, Schmalz G, Brockhoff G, Reichert TE, Morsczeck C: The transcription factor DLX3 regulates the osteogenic differentiation of human dental follicle precursor cells. Stem Cells Dev 2012;21: 1936–1947.
Vieira AR, Gibson CW, Deeley K, Xue H, Li Y: Weaker dental enamel explains dental decay. PLoS One 2015;10:e0124236.
Vieira AR, Marazita ML, Goldstein-McHenry T: Genome-wide scan finds suggestive caries loci. J Dent Res 2008;87:435–439.
Volckova M, Linhartova PB, Trefna T, Vlazny J, Musilova K, Kukletova M, Kukla L, Holla LI: Lack of association between lactotransferrin polymorphism and dental caries. Caries Res 2014;48:39–44.
Wang RN, Green J, Wang Z, Deng Y, Qiao M, Peabody M, Zhang Q, Ye J, Yan Z, Denduluri S, Idowu O, Li M, Shen C, Hu A, Haydon RC, Kang R, Mok J, Lee MJ, Luu HL, Shi LL: Bone morphogenetic protein (BMP) signaling in development and human diseases. Genes Dis 2014;1:87–105.
Wang X, Shaffer JR, Weyant RJ, Cuenco KT, DeSensi RS, Crout R, McNeil DW, Marazita ML: Genes and their effects on dental caries may differ between primary and permanent dentitions. Caries Res 2010;44:277–284.
Wu M, Chen G, Li YP: TGF-β and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. Bone Res 2016;4:16009.
Zhang Z, Tian H, Lv P, Wang W, Jia Z, Wang S, Zhou C, Gao X: Transcriptional factor DLX3 promotes the gene expression of enamel matrix proteins during amelogenesis. PLoS One 2015;10:e0121288.

Downloaded by:
Masaryk University
147.251.6.77 - 10/24/2017 10:17:05 AM

Attachment 9 Article ”Lack of association between *ENAM* gene polymorphism and dental caries in primary and permanent teeth in Czech children”

Lack of association between ENAM *gene polymorphism and dental caries in primary and permanent teeth in Czech children*

Petra Borilova Linhartova, Tereza Deissova, Kristina Musilova, Lenka Zackova, Martina Kukletova, Lubomir Kukla & Lydie Izakovicova Holla

Clinical Oral Investigations

ISSN 1432-6981
Volume 22
Number 4

Clin Oral Invest (2018) 22:1873-1877
DOI 10.1007/s00784-017-2280-2

Volume 22 · Number 4 · May 2018

Clinical Oral Investigations

4
2018

Springer

Springer

Clinical Oral Investigations (2018) 22:1873–1877
https://doi.org/10.1007/s00784-017-2280-2

SHORT COMMUNICATION

Lack of association between *ENAM* gene polymorphism and dental caries in primary and permanent teeth in Czech children

Petra Borilova Linhartova [1,2] · **Tereza Deissova** [1] · **Kristina Musilova** [1] · **Lenka Zackova** [3] · **Martina Kukletova** [1] · **Lubomir Kukla** [4] · **Lydie Izakovicova Holla** [1,2,4]

Received: 27 February 2017 / Accepted: 20 November 2017 / Published online: 28 November 2017
© Springer-Verlag GmbH Germany, part of Springer Nature 2017

Abstract

Objectives The *enamelin* gene (*ENAM*) polymorphism (rs12640848) was recently associated with dental caries in primary teeth in Polish children. The aims of the present study were to prove this association in primary dentition and to find a possible effect of this variant on caries development in permanent dentition in Czech children.

Materials and methods This study comprised 905 Czech children. Totally, 187 children aged 2–6 years with primary dentition [78 healthy subjects (with decayed/missing/filled teeth, dmft = 0) and 109 patients with early childhood caries (ECC; dmft ≥ 1)] were included in this case-control study. In addition, 177 subjects aged 13–15 years without caries (DMFT = 0) and 541 children with dental caries (DMFT ≥ 1) in permanent dentition were selected from the ELSPAC study. Genotype determination of the *ENAM* polymorphism (rs12640848) was based on the TaqMan method.

Results No significant differences in the allele or genotype frequencies between the caries-free children and those affected by dental caries were observed in both primary and permanent dentitions.

Conclusions Lack of association between the *ENAM* polymorphism (rs12640848) and dental caries in Czech children was detected.

Clinical relevance Although *ENAM* is considered as a candidate gene for dental caries, the presence of the *ENAM* variant (rs12640848) cannot be used as a risk factor of this multifactorial disease in the Czech population.

Keywords Enamelin · Gene polymorphism · Dental caries · Case-control study · Primary dentition · Permanent dentition

Introduction

Dental caries which affects the mineralized tissues of teeth (enamel, dentin, and cementum) is one of the most common multifactorial diseases. Early childhood caries (ECC) is then defined as the presence of at least one decayed tooth (cavitated or non-cavitated), missing tooth (due to caries), or filled dental surfaces (due to caries) in any primary tooth in children aged

✉ Lydie Izakovicova Holla
holla@med.muni.cz

Petra Borilova Linhartova
plinhart@med.muni.cz

Tereza Deissova
436741@mail.muni.cz

Kristina Musilova
51179@mail.muni.cz

Lenka Zackova
lenkazackova@seznam.cz

Martina Kukletova
makukl@med.muni.cz

Lubomir Kukla
30125@mail.muni.cz

[1] Clinic of Stomatology, Institution Shared with St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Pekarska 664/53, 60200 Brno, Czech Republic

[2] Department of Pathophysiology, Faculty of Medicine, Masaryk University, Kamenice 5, 62500 Brno, Czech Republic

[3] Stomatology Research Center, Faculty of Medicine, Masaryk University, Komenskeho namesti 220/2, 602 00 Brno, Czech Republic

[4] Research Centre for Toxic Compounds in the Environment (RECETOX), Chemistry Section, Faculty of Science, Masaryk University, Kamenice 5, 62500 Brno, Czech Republic

6 years or younger [1]. Results of longitudinal and cross-sectional studies suggest that caries experience in the primary dentition may have an adverse effect on caries polarization in adolescence [2, 3].

Clinically, progression of carious lesions differs between the primary and permanent dentitions; this results from the morphological differences of both dentitions. Wang et al. [4] found that primary enamel demineralized considerably faster than permanent, which is the critical factor in the caries development [5]. Although amelogenin is the most abundant protein in developing enamel, the expression of other proteins such as ameloblastin and enamelin was found by liquid-chromatography coupled to tandem mass spectrometry [6]. However, the process of enamel formation may be influenced by many factors (fever, malnutrition, xenobiotic and pollutant exposure, etc.), including genetic component.

Thinner dental enamel has been associated with the specific *ENAM* genotype [7]. Nevertheless, variability in the *ENAM* gene in relation to dental caries in primary and/or permanent dentition was studied with contradictory results [8–17]. Bayram at el. [18] reported that the *ENAM* variants may impact enamel development and these effects may be distinct between primary and permanent dentitions.

The *ENAM* polymorphism (rs12640848) has recently been associated with ECC in Polish [19] and Turkish [20] children. In addition, Shaffer et al. [21] found an association of this single-nucleotide polymorphism (SNP) with dental caries in permanent dentition using meta-analysis across white adult samples. The aims of the present study were (i) to prove the association between the *ENAM* SNP (rs12640848) and ECC and (ii) to find a possible effect of this variant on caries development in permanent dentition in Czech children.

Material and methods

Subjects and clinical examinations

During the period of 2005 to 2011, 109 preschool Czech children with ECC (64 boys and 45 girls), aged 2–6 years, were recruited for complex treatment under general anesthesia. All children were generally healthy but unable to undergo a standard dental treatment due to their uncooperativeness and a need for multiple restorations and extractions. Oral examination was performed and described previously [22]. In 2011, dental status of 78 generally healthy children aged 2–6 years was examined. The primary dentition of these young children (average age ± standard deviation: 3.8 ± 1.1, 36 boys and 42 girls) was caries-free.

In addition, a total of 718 unrelated Caucasian children with permanent dentition (379 boys and 339 girls), aged 13–15 years, selected from the ELSPAC Brno study comprising over 5000 children and their families [23, 24] were investigated. These children underwent dental examination at the Clinic of Stomatology at St. Anne's University Hospital in Brno as described previously [25]. The study group comprised caries-affected subjects (with decayed/missing/filled teeth (DMFT) ≥ 1; N = 541; 285 boys and 256 girls) and caries-free children (with DMFT = 0; N = 177; 94 boys and 83 girls).

The dmft/DMFT indexes were calculated using dentinal caries (D_3 level) as a *cutoff point* for the detection of decay.

SNP genotyping TaqMan® assay

DNA for genetic analysis was isolated from buccal epithelial cells (children from the ELSPAC study and from young caries-free children) or from blood samples (young children with ECC). Extractions were performed according to the manufacturer's instructions for the Ultra-Clean®BloodSpin® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, Calif., USA) with small adaptations.

Genotyping of the *ENAM* SNP (rs12640848) was based on polymerase chain reaction using 5′ nuclease TaqMan®assay (C___3065260_20). The reaction mixture and conditions were designed according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA) and fluorescence was measured using ABI PRISM 7000 Sequence Detection System. SDS version 1.2.3 software was used to analyze real-time and endpoint fluorescence data. Genotyping was verified by using positive control subjects in each 96-well plate and rerunning ≥ 5% of the samples, which were 100% concordant.

Statistical analysis

All statistical analyses were performed using the program package Statistica v. 12.0 (StatSoft, Inc., Tulsa, OK, USA). The chi-square and Fisher's exact tests were used for comparison of differences in genotype or allele frequencies among the groups.

Results and discussion

Etiology of dental caries has been related to a combination of environmental risk factors (oral hygiene, diet, fluoride exposure, etc.) and genetic predisposition [26, 27]. One of the most studied groups of candidate genes includes the enamel formation genes, such as *ENAM* [28]. Results of all previous studies on the *ENAM* SNP (rs12640848) are summarized in Table 1.

In this study, which comprised 905 Czech children, no differences in the allele or genotype frequencies of the *ENAM* SNP (rs12640848) between caries-affected and caries-free Czech children with primary or permanent dentitions were found, see Table 2. In addition, no significant

Table 1 Review of genetic studies focused on the analysis of *ENAM* SNP (rs12640848)

Reference	Population	*N* of cases/controls	Dentition (age of subjects)	MAF	Significance
[19]	Polish (Caucasians)	48/48[a]	Primary (20–48 months)	0.45 (A)	G allele and GG genotype protective for ECC
[20]	Turkish	136/123[a]	Primary (2–5 years)	0.36	GG genotype protective for ECC (multivariate analysis gene environment)
[21] (included data from [13])	13 race- and age-stratified samples (non-Hispanic whites and blacks)	480/409[a](children) 2601/97[a] (adults)	Primary (3–12 years) Permanent (15–79 years)	0.37 (A)	NS (association via meta-analysis across white adults)
[16]	Turkish	100/100	Mixed (6–12 years)	0.22	NS
[14]	Turkish	136/82	Both (8–11 years)	0.47 (A)	NS
	Brazilian	71/89[b]	Both (5–20 years)	0.49 (G)	NS (association with caries after logistic regression analysis)
[13]	Americans (95% from Caucasian families, 2% African American, 3% others)	82/251[a] (children and parents, Caucasians)	Primary (4–7 years, Caucasians) Permanent (in parents, Caucasians)	0.33 (A, Caucasians)	NS
[12]	Turkish	92/80[b]	Primary (3–6 years)	–	NS
	Filipino	298/179[c]	Permanent (12 years and older, families)	0.20 (G)	
	Argentinean	66/77[d]	Both types (1–72 years)	–	
	Brazilian (Rio de Janeiro)	171/329[d]	Both types (2–21 years)	–	
	Brazilian (Curitiba)	117/410[d]	Permanent (10–14 years)	0.34 (A)	A allele is risk for dental caries

ECC early childhood caries, *ENAM* enamelin, *dmft/DMFT* decayed/missing/filled teeth in primary/permanent dentitions, *MAF* minor allele frequency, *N* number of subjects, *NS* non-significant, *SNP* single-nucleotide polymorphism

[a] Controls dmft/DMFT = 0, cases dmft/DMFT ≥ 1

[b] Controls 0 ≤ dmft ≤ 3, cases dmft ≥ 4

[c] Stratification on the low (0 ≤ DMFT ≤ 2/5/8) and high caries experience groups according to the age of subjects (up to 12 years/from 13 to 19 years/20 years and older, respectively)

[d] Controls 0 ≤ DMFT ≤ 2, cases DMFT ≥ 3

differences between children with DMFT = 0 and DMFT ≥ 5 were detected.

Our results correspond with the findings reported by Wang et al. [13] (included in the study by Shaffer et al. [21]), Jeremias et al. [14], and Ergözet et al. [16]. Similar conclusions were confirmed by Shimizu et al. [12] in the studied populations with the exception of the Brazilian population from Curitiba where the A allele was associated with dental caries development in permanent dentition.

Similarly, the G allele and/or GG genotype were protective for ECC in the Polish and Turkish children [19, 20]. Gerreth et al. [19] reported 45% frequency of the G allele in children with ECC versus 65% in caries-free children in primary dentition. Although both the Poles and Czechs are European Caucasians, there are differences in the minor allele frequency (MAF) in *ENAM* SNP (rs12640848) between these populations. Disagreements between the allele distributions in the Polish and Czech population in another gene have also been described previously [11, 22]. The A allele *ENAM* SNP (rs12640848) was found in 45% of Polish children but only in 33% of Czech subjects, which is in line with the MAF (0.33) in European Caucasians referring to the NCBI database (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_viewTable.cgi?pop=16652). Interestingly, the G allele is the minor allele in other populations (Asians, Africans, Americans), except Brazilians (Curitiba) [12].

Interpopulation differences may be the reason for the controversial results of the case-control association studies.

Table 2 Distribution of the *ENAM* SNP (rs12640848) genotype and allele frequencies in children with ECC/dental caries (dmft/DMFT ≥ 1 or DMFT ≥ 5) versus the caries-free children in primary/permanent dentition (dmft/DMFT = 0)

Gene polymorphism	Caries-free children (dmft = 0)	Children with ECC (dmft ≥ 1)	Caries-free children (DMFT = 0)	Caries-affected children (DMFT ≥ 1)	Caries-affected children (DMFT ≥ 5)
ENAM (rs12640848)	*N* = 78 (%)	*N* = 109 (%)	*N* = 177 (%)	*N* = 541 (%)	*N* = 174 (%)
GG	28 (35.9)	46 (42.2)	84 (47.5)	237 (43.8)	75 (43.1)
AG	46 (59.0)	50 (45.9)	74 (41.8)	259 (47.9)	84 (48.3)
AA	4 (5.1)	13 (11.9)	19 (10.7)	45 (8.3)	15 (8.6)
G allele	102 (65.4)	142 (65.1)	242 (68.4)	733 (67.7)	234 (67.2)
A allele	54 (34.6)	76 (34.9)	112 (31.6)	349 (32.3)	114 (32.8)

ECC early childhood caries, *ENAM* enamelin, *dmft/DMFT* decayed/missing/filled teeth, *N* number of subjects

Nevertheless, there is evidence that specific genetic variations of enamel formation genes may have an impact on the development of caries, for example by influencing calcium and magnesium concentrations of teeth [29]. Haplotype analysis by Chaussain et al. [15] supported the presumption that *ENAM* was a gene candidate for dental caries susceptibility in permanent dentition in French population. However, the *ENAM* SNP (rs12640848) was not analyzed in their study.

Two main limitations of this study must be considered. Firstly, only one polymorphism in the *ENAM* gene was studied and thus the haplotype analysis could not be performed. Secondly, we have no information regarding the presence of *Streptococcus mutans*, a major bacterium related with dental caries. Strength of our study is a relatively large sample size and homogeneity of the population sample. Considering the fact that genetic susceptibility to caries can differ between primary and permanent teeth [22, 30], children with both dentitions were included in this study.

In conclusion, no association between the *ENAM* gene polymorphism (rs12640848) and dental caries in primary and permanent teeth in Czech children was found. Although *ENAM* is considered as a candidate gene for dental caries, we assume that individual variations in genes have little effect and complex approach in the research of multifactorial disease is necessary.

Authors' contributions PBL, MK, LK, and LIH designed the study and drafted the paper. KM, LZ, and MK performed the clinical analyses and collected blood or saliva samples. PBL and TD carried out the molecular analysis. LIH performed statistical analysis. All authors approved the final version of the manuscript.

Funding The study was supported by funds provided by the Faculty of Medicine MU to junior researcher Petra Borilova Linhartova, grants AZV 17-30439A, GACR GB14-37368G, projects MUNI/A/0948/2016 and Recetox RI (CZ.02.1.01/0.0/0.0/16_013/0001761).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by the Committee for Ethics of the Faculty of Medicine, MU Brno (3/2004, from 30/03/ 2004) and St. Anne's Faculty Hospital (without number, from 13/04/2004 and 1G/2017).

Informed consent In line with the Helsinki declaration, informed consent was obtained from all participants or legal representatives of children included in the study.

References

1. American Academy of Peadiatric Dentistry (2014) Policy on early childhood caries (ECC): classifications, consequences, and preventive strategies. http://www.aapd.org/media/Policies_Guidelines/P_ECCClassifications.pdf. Accessed 19 Feb 2015
2. Skeie MS, Raadal M, Strand GV, Espelid I (2006) The relationship between caries in the primary dentition at 5 years of age and permanent dentition at 10 years of age—a longitudinal study. Int J Paediatr Dent 16(3):152–160. https://doi.org/10.1111/j.1365-263X.2006.00720.x
3. Zemaitiene M, Grigalauskiene R, Andruskeviciene V, Matulaitiene ZK, Zubiene J, Narbutaite J, Slabsinskiene E (2016) Dental caries risk indicators in early childhood and their association with caries polarization in adolescence: a cross-sectional study. BMC Oral Health 17(1):2. https://doi.org/10.1186/s12903-016-0234-8
4. Wang LJ, Tang R, Bonstein T, Bush P, Nancollas GH (2006) Enamel demineralization in primary and permanent teeth. J Dent Res 85(4):359–363. https://doi.org/10.1177/154405910608500415
5. Vieira AR, Gibson CW, Deeley K, Xue H, Li Y (2015) Weaker dental enamel explains dental decay. PLoS One 10(4):e0124236. https://doi.org/10.1371/journal.pone.0124236
6. Castiblanco GA, Rutishauser D, Ilag LL, Martignon S, Castellanos JE, Mejia W (2015) Identification of proteins from human permanent erupted enamel. Eur J Oral Sci 123(6):390–395. https://doi.org/10.1111/eos.12214
7. Daubert DM, Kelley JL, Udod YG, Habor C, Kleist CG, Furman IK, Tikonov IN, Swanson WJ, Roberts FA (2016) Human enamel thickness and ENAM polymorphism. Int J Oral Sci 8(2):93–97. https://doi.org/10.1038/ijos.2016.1
8. Slayton RL, Cooper ME, Marazita ML (2005) Tuftelin, mutans streptococci, and dental caries susceptibility. J Dent Res 84(8):711–714. https://doi.org/10.1177/154405910508400805
9. Deeley K, Letra A, Rose EK, Brandon CA, Resick JM, Marazita ML, Vieira AR (2008) Possible association of amelogenin to high caries experience in a Guatemalan-Mayan population. Caries Res 42(1):8–13. https://doi.org/10.1159/000111744

10. Patir A, Seymen F, Yildirim M, Deeley K, Cooper ME, Marazita ML, Vieira AR (2008) Enamel formation genes are associated with high caries experience in Turkish children. Caries Res 42(5):394–400. https://doi.org/10.1159/000154785
11. Olszowski T, Adler G, Janiszewska-Olszowska J, Safranow K, Kaczmarczyk M (2012) MBL2, MASP2, AMELX, and ENAM gene polymorphisms and dental caries in Polish children. Oral Dis 18(4):389–395. https://doi.org/10.1111/j.1601-0825.2011.01887.x
12. Shimizu T, Ho B, Deeley K, Briseño-Ruiz J, Faraco IM Jr, Schupack BI, Brancher JA, Pecharki GD, Küchler EC, Tannure PN, Lips A, Vieira TC, Patir A, Yildirim M, Poletta FA, Mereb JC, Resick JM, Brandon CA, Orioli IM, Castilla EE, Marazita ML, Seymen F, Costa MC, Granjeiro JM, Trevilatto PC, Vieira AR (2012) Enamel formation genes influence enamel microhardness before and after cariogenic challenge. PLoS One 7(9):e45022. https://doi.org/10.1371/journal.pone.0045022
13. Wang X, Willing MC, Marazita ML, Wendell S, Warren JJ, Broffitt B, Smith B, Busch T, Lidral AC, Levy SM (2012) Genetic and environmental factors associated with dental caries in children: the Iowa Fluoride Study. Caries Res 46(3):177–184. https://doi.org/10.1159/000337282
14. Jeremias F, Koruyucu M, Küchler EC, Bayram M, Tuna EB, Deeley K, Pierri RA, Souza JF, Fragelli CM, Paschoal MA, Gencay K, Seymen F, Caminaga RM, dos Santos-Pinto L, Vieira AR (2013) Genes expressed in dental enamel development are associated with molar-incisor hypomineralization. Arch Oral Biol 58(10):1434–1442. https://doi.org/10.1016/j.archoralbio.2013.05.005
15. Chaussain C, Bouazza N, Gasse B, Laffont AG, Opsahl Vital S, Davit-Béal T, Moulis E, Chabadel O, Hennequin M, Courson F, Droz D, Vaysse F, Laboux O, Tassery H, Carel JC, Alcais A, Treluyer JM, Beldjord C, Sire JY (2014) Dental caries and enamelin haplotype. J Dent Res 93(4):360–365. https://doi.org/10.1177/0022034514522060
16. Ergöz N, Seymen F, Gencay K, Tamay Z, Deeley K, Vinski S, Vieira AR (2014) Genetic variation in ameloblastin is associated with caries in asthmatic children. Eur Arch Paediatr Dent 15(3):211–216. https://doi.org/10.1007/s40368-013-0096-6
17. Søvik JB, Vieira AR, Tveit AB, Mulic A (2015) Enamel formation genes associated with dental erosive wear. Caries Res 49(3):236–242. https://doi.org/10.1159/000369565
18. Bayram M, Deeley K, Reis MF, Trombetta VM, Ruff TD, Sencak RC, Hummel M, Dizak PM, Washam K, Romanos HF, Lips A, Alves G, Costa MC, Granjeiro JM, Antunes LS, Küchler EC, Seymen F, Vieira AR (2015) Genetic influences on dental enamel that impact caries differ between the primary and permanent dentitions. Eur J Oral Sci 123(5):327–334. https://doi.org/10.1111/eos.12204 [Epub ahead of print]
19. Gerreth K, Zaorska K, Zabel M, Borysewicz-Lewicka M, Nowicki M (2016) Association of ENAM gene single nucleotide polymorphisms with dental caries in Polish children. Clin Oral Investig 20(3):631–636. https://doi.org/10.1007/s00784-016-1743-1
20. Abbasoğlu Z, Tanboğa İ, Küchler EC, Deeley K, Weber M, Kaspar C, Korachi M, Vieira AR (2015) Early childhood caries is associated with genetic variants in enamel formation and immune response genes. Caries Res 49(1):70–77. https://doi.org/10.1159/000362825
21. Shaffer JR, Carlson JC, Stanley BO, Feingold E, Cooper M, Vanyukov MM, Maher BS, Slayton RL, Willing MC, Reis SE, McNeil DW, Crout RJ, Weyant RJ, Levy SM, Vieira AR, Marazita ML (2015) Effects of enamel matrix genes on dental caries are moderated by fluoride exposures. Hum Genet 134(2):159–167. https://doi.org/10.1007/s00439-014-1504-7
22. Borilova Linhartova P, Kastovsky J, Bartosova M, Musilova K, Zackova L, Kukletova M, Kukla L, Izakovicova Holla L (2016) ACE insertion/deletion polymorphism associated with caries in permanent but not primary dentition in Czech children. Caries Res 50(2):89–96. https://doi.org/10.1159/000443534
23. No authors listed (1989) European Longitudinal Study of Pregnancy and Childhood (ELSPAC). Paediatr Perinat Epidemiol 3:460–469.
24. Piler P, Kandrnal V, Kukla L, Andryskova L, Svancara J, Jarkovsky J, Dusek L, Pikhart H, Bobak M, Klanová J (2016) Cohort profile: the European Longitudinal Study of Pregnancy and Childhood (ELSPAC) in the Czech Republic. Int J Epidemiol 46(5):1379–1379f. https://doi.org/10.1093/ije/dyw091
25. Volckova M, Borilova Linhartova P, Trefna T, Vlazny J, Musilova K, Kukletova M, Kukla L, Izakovicova Holla L (2014) Lack of association between lactotransferrin polymorphism and dental caries. Caries Res 48(1):39–44. https://doi.org/10.1159/000351689
26. Vieira AR, Modesto A, Marazita ML (2014) Caries: review of human genetics research. Caries Res 48(5):491–506. https://doi.org/10.1159/000358333
27. Opal S, Garg S, Jain J, Walia I (2015) Genetic factors affecting dental caries risk. Aust Dent J 60(1):2–11. https://doi.org/10.1111/adj.12262
28. Selwitz RH, Ismail AI, Pitts NB (2007) Dental caries. Lancet 369(9555):51–59. https://doi.org/10.1016/S0140-6736(07)60031-2
29. Halusic AM, Sepich VR, Shirley DC, Granjeiro JM, Costa MC, Küchler EC, Vieira AR (2014) Calcium and magnesium levels in primary tooth enamel and genetic variation in enamel formation genes. Pediatr Dent 36(5):384–388
30. Wang X, Shaffer JR, Weyant RJ, Cuenco KT, De-Sensi RS, Crout R, McNeil DW, Marazita ML (2010) Genes and their effects on dental caries may differ between primary and permanent dentitions. Caries Res 44(3):277–284. https://doi.org/10.1159/000314676

Attachment 10 Article "*GLUT2* and *TAS1R2* polymorphisms and susceptibility to dental caries"

Original Paper

Caries Research

Caries Res 2015;49:417–424
DOI: 10.1159/000430958

Received: February 26, 2015
Accepted after revision: April 26, 2015
Published online: June 24, 2015

GLUT2 and *TAS1R2* Polymorphisms and Susceptibility to Dental Caries

Lydie Izakovicova Holla[a, b] Petra Borilova Linhartova[a, b] Svetlana Lucanova[a, c] Jakub Kastovsky[a, c] Kristina Musilova[a] Michaela Bartosova[a, d] Martina Kukletova[a] Lubomir Kukla[d] Ladislav Dusek[e]

[a]Clinic of Stomatology, Institutions Shared with St. Anne's Faculty Hospital, and [b]Department of Pathophysiology, Faculty of Medicine, [c]Faculty of Science, [d]RECETOX, Research Centre for Toxic Compounds in the Environment, and [e]Institute of Biostatistics and Analyses, Masaryk University, Brno, Czech Republic

Key Words

Case-control study · Dental caries · Gene polymorphisms · *GLUT2* · *TAS1R2*

Abstract

Objective: Dental caries is one of the most frequent multifactorial diseases. Among the numerous factors influencing the risk of caries, genetics plays a substantial role, with heritability ranging from 40 to 60%. Gene variants affecting taste preference and glucose transport were recently associated with caries risk. The aim of this study was to analyze two common polymorphisms in the sweet taste receptor *(TAS1R2)* and glucose transporter *(GLUT2)* genes in children with dental caries and healthy controls in the Czech population. ***Methods:*** A total of 637 unrelated Caucasian children, aged 11–13 years, were included in this case-control study. One hundred and fifty-five subjects were caries-free (with decayed/missing/filled teeth, DMFT = 0) and 482 children were caries-affected (DMFT ≥ 1). The *TAS1R2* (Ile191Val, rs35874116) and *GLUT2* (Thr110Ile, rs5400) genotypes were determined using the 5′ nuclease TaqMan® assay for allelic discrimination. ***Results:*** Compared with subjects with the common Thr allele, carriers of the Ile allele of *GLUT2* had significantly more frequently dental caries ($p < 0.05$, OR = 1.639, 95% CI: 1.089–2.466). Similarly, children with the Val allele for the *TAS1R2* Ile191Val polymorphism were more frequently affected by caries than children who carried the Ile allele ($p < 0.05$, OR = 1.413, 95% CI: 1.014–1.969). In contrast, no significant associations between *GLUT2* and/or *TAS1R2* polymorphisms and fillings were found, but allele frequencies of the *TAS1R2* variant were marginally significantly different between children with DMFT = 0 and DMFT ≥1 ($p = 0.053$, OR = 1.339, 95% CI: 0.996–1.799). However, no significant interaction between both genes and risk of dental caries was found. ***Conclusions:*** In conclusion, *GLUT2* and *TASR1* polymorphisms may influence the risk of caries in the Czech population.
© 2015 S. Karger AG, Basel

Dental caries is one of the most prevalent chronic diseases worldwide. It results from the interaction of specific microflora with fermentable carbohydrate on a susceptible tooth surface. The relationship between caries and saliva, host response, fluoride and other factors has been intensively investigated during the last years [Slayton et al., 2005]. Among the numerous factors influencing risk for caries, genetics plays a substantial role, with

S.L. and J.K. contributed equally to this work.

KARGER 125

© 2015 S. Karger AG, Basel
0008–6568/15/0494–0417$39.50/0

E-Mail karger@karger.com
www.karger.com/cre

Prof. Lydie Izakovicova Holla, MD, PhD
Clinic of Stomatology and Department of Pathophysiology
Medical Faculty, Masaryk University, Pekarska 53
CZ–656 91 Brno (Czech Republic)
E-Mail holla@med.muni.cz

Downloaded by: Masaryk University 195.113.50.1 - 10/20/2016 9:27:22 AM

heritability ranging from 40 to 60% [Wang et al., 2010]. Genes related to tooth morphology and enamel formation, saliva flow and composition, and also dietary and taste preferences have recently been identified [Slayton et al., 2005; Deeley et al., 2008; Patir et al., 2008; Kang et al., 2011; Tannure et al., 2012a, b; Kulkarni et al., 2013].

The sweet taste receptor is a heterodimer of two protein subunits, T1R2 (taste receptor type 1, member 2) and T1R3 (taste receptor type 1, member 3), that are encoded by the *TAS1R2* and *TAS1R3* genes located on human chromosome 1 [Liao and Schultz, 2003]. The diverse tissue distribution of the sweet taste receptor places *TAS1R2* as a candidate gene affecting food intake beyond the detection of sweet taste on the tongue and palate [Nelson et al., 2001]. Although *TAS1R2* is highly polymorphic, there are only two common polymorphisms with minor allele frequencies above 10%. One of them, Ile191Val (rs35874116), is located in one of the putative ligand binding sites of this protein [Kim et al., 2006] and was associated with a lower habitual sugar intake [Eny et al., 2010]. GLUT2, encoded by the *GLUT2 (SLC2A2)* gene, is a member of the glucose transport protein family that is expressed in many tissues, including brain. *GLUT2* expression has been localized to regions of the brain involved in food intake regulation in humans [Roncero et al., 2004]. A common single-nucleotide polymorphism (SNP) results in a threonine-to-isoleucine amino acid substitution at codon 110 (Thr110Ile, rs5400). The Thr110Ile variant did not show any decrease in transport activity [Mueckler et al., 1994], but it has recently been found that carriers of the Ile allele have a significantly higher intake of sugars than individuals with the Thr/Thr genotype in a Canadian population [Eny et al., 2008]. To date, only one study has analyzed allele and genotype frequencies of both *GLUT2* and *TAS1R2* polymorphisms in patients with dental caries, and the authors found a higher DMFT (decayed/missing/filled teeth) score in carriers of the Ile allele for *GLUT2* (risk group) and a lower caries score in carriers of the Val allele for *TAS1R2* (resistant group). In addition, caries scores were significantly lower in the double-resistant group as compared to the double-risk group in a Canadian adult population [Kulkarni et al., 2013].

Because diet with a high consumption of sugar is an important contributing risk factor for caries and evidence suggests genetically determined dietary preferences, we hypothesized that polymorphisms in both *GLUT2* and *TAS1R2* genes might modify the relative risk for dental caries development also in Czech children. Therefore, the aims of the present study were to compare the distribution of the *GLUT2* and *TAS1R2* alleles and genotypes between Czech children with and without caries and to examine whether these gene variants were associated with caries experience as measured by the DMFT index.

Methods

Subjects

The total number of 637 unrelated Caucasian children (324 boys and 313 girls), aged 11–13 years, selected from the European Longitudinal Study of Pregnancy and Childhood (ELSPAC) [1989] Brno study comprising over 5,000 children and their families [Kukla et al., 1992] were investigated. Children underwent dental examination at the Clinic of Stomatology at St. Anne's University Hospital and the Faculty of Medicine, Masaryk University, as described previously [Volckova et al., 2014]. The study group (n = 482; 242 boys and 240 girls) comprised caries-affected children. The healthy group (n = 155; 82 boys and 73 girls) included caries-free children.

Clinical Examinations

The clinical assessment was carried out by one investigator using the following clinical parameters: the cavitation of lesions as the detection threshold of caries according to the criteria given in the WHO Oral Health Surveys, 4th edition [1997], gingival index, plaque index and calculus index as described previously [Holla et al., 2008]. Radiography was not performed as it was not part of routine dental care for these adolescents and would therefore be deemed unethical. The phenotype status was assigned without knowledge of genotypes by two independent investigators (K.M. and M.K.).

The study was approved by the Committee for Ethics of the Faculty of Medicine, Masaryk University, Brno and informed consent was obtained from all parents (in case of children), in line with the Helsinki Declaration before inclusion in the study.

DNA Isolation

DNA for genetic analysis was isolated from buccal epithelial cells obtained by buccal swabs. Extractions were performed according to the manufacturer's instructions for the UltraClean® BloodSpin® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, Calif., USA) with slight adaptations.

SNP Genotyping TaqMan® Assay

Polymorphisms in the *TAS1R2* (Ile191Val, rs35874116) and *GLUT2* genes (Thr110Ile, rs5400) were genotyped using the 5′ nuclease TaqMan® assay for allelic discrimination (Life Technologies, Grand Island, N.Y., USA). Details on SNP detection are summarized in table 1. PCR conditions were 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min as described previously [Eny et al., 2008, 2010]. Allele genotyping from fluorescence measurements was then obtained using the ABI PRISM 7000 Sequence Detection System. SDS version 1.2.3 software was used to analyze real-time and endpoint fluorescence data. Genotyping was performed by three investigators (S.L., J.K. and P.B.L.) unaware of the phenotype. Genotyping was verified by using positive control subjects in each 96-well plate and rerunning ≥5% of the samples, which were 100% concordant.

Downloaded by:
Masaryk University
198.143.55.1 - 10/26/2015 9:27:22 AM

Table 1. Details of SNPs in *GLUT2* and *TAS1R2* detection

SNPs	TaqMan® SNP genotyping assay ID	Context sequence [VIC/FAM]	Genotypes
rs5400 *GLUT2* Thr110Ile	C_3142148_10 forward assay	AAGCCACCCACCAAAGAATGATGCA[A/G] TCATTCCACCAACTGCAAAGCTGGA	AA (Ile/Ile) AG (Ile/Thr) GG (Thr/Thr)
rs35874116 *TAS1R2* Ile191Val	C_55646_20 forward assay	AGCATCAGCTGCACCATGGCCTCGA[C/T] GTGGTGGTCGGCGCTGGGTGTGGTA	CC (Val/Val) CT (Val/Ile) TT (Ile/Ile)

Statistical Analysis

Standard descriptive statistics were applied in the analysis: absolute and relative frequencies for categorical variables and mean, median and min-max range for quantitative variables. Statistical significance of association between endpoints and genes was computed by means of Fisher's exact test; the association was described by odds ratios supplemented by their 95% confidence intervals computed using logistic regression. The power analysis was computed for the association analysis between the genes and caries, using the model of the most risk genotype AA. The analysis was computed using SPSS 22 (IBM Corp., 2013).

Results

Of 637 individuals included in this study, 155 (24.3%) were caries-free and 482 (75.6%) were carries-affected; 103 (21.4%) of them were classified as children with low (DMFT = 1), 158 (32.8%) with moderate (2≤ DMFT ≤3), and 221 (45.6%) with high (DMFT ≥4) caries experience. Statistical power for the association between the observed frequency of the genotype in patients with caries versus subjects with DMFT = 0 was 0.769.

Table 2 shows a basic description of the sample. Boys and girls aged 11–13 years ($p > 0.05$) were similarly distributed. The mean DMFT value was 2.92 in the whole set; on average, the children had 0.64 caries, 2.28 fillings and extraction was only performed in 3 of them; 27.8% were carriers of the minor (Ile) allele for the Thr110Ile polymorphism of *GLUT2* and 48% were carriers of the Val allele for the Ile191Val polymorphism of *TAS1R2*.

The relationships between *GLUT2* and *TAS1R2* polymorphisms and caries in children with and without caries (DMFT = 0) are shown in table 3a. The caries-free group was in Hardy-Weinberg equilibrium with nonsignificant χ^2 values comparing the observed and expected genotype frequencies of both investigated variants. Compared with the subjects with the common Thr allele, the carriers of the Ile allele of the *GLUT2* gene had significantly more

Table 2. Basic description of the sample

	Characteristics[1]
Patient-based analysis (n = 637)	
Gender	
Boys	324 (50.9)
Girls	313 (49.1)
rs5400 *GLUT2* Thr110Ile	
AA	21 (3.3)
AG	156 (24.5)
GG	460 (72.2)
A allele	177 (27.8)
G allele	616 (96.7)
rs35874116 *TAS1R2* Ile191Val	
CC	53 (8.3)
CT	253 (39.7)
TT	331 (52.0)
C allele	306 (48.0)
T allele	584 (91.7)
Number of carious teeth	0.64; 0 (0; 11.0)
Caries	
No	422 (66.2)
Yes	215 (33.8)
Number of fillings	2.28; 2 (0; 12.0)
Fillings	
No	213 (33.4)
Yes	424 (66.6)
Number of extractions	0.00; 0 (0; 1.0)
Extractions	
No	634 (99.5)
Yes	3 (0.5)
DMFT score	2.92; 2 (0; 19.0)
DMFT	
0	155 (24.3)
≥1	482 (75.7)
Allele-based analysis (n = 1,274)	
rs5400 *GLUT2* Thr110Ile	
A allele	198 (15.5)
G allele	1,076 (84.5)
rs35874116 *TAS1R2* Ile191Val	
C allele	359 (28.2)
T allele	915 (71.8)

Figures in parentheses are percentages.
[1] Absolute and relative frequencies for categorical variables; mean, median and min-max for count variables.

Downloaded by: Masaryk University 198.143.55.1 - 10/20/2015 9:27:22 AM

Table 3. Relationship between caries, fillings, DMFT and genes

a Relationship between caries and genes

	Endpoint[1]		p[2]	OR (95% CI)[3]	p[3]
	DMFT = 0	caries: yes			
Patient-based analysis	(n = 155)	(n = 215)			
Gender					
Boys	82 (52.9)	115 (53.5)	0.916	reference	–
Girls	73 (47.1)	100 (46.5)		0.977 (0.646–1.478)	0.911
rs5400 *GLUT2* Thr110Ile					
AA	2 (1.3)	12 (5.6)	0.041	4.909 (1.077–22.373)	0.040
AG	36 (23.2)	60 (27.9)		1.364 (0.844–2.204)	0.205
GG	117 (75.5)	143 (66.5)		reference	
A allele					
No	117 (75.5)	143 (66.5)	0.066	reference	–
Yes	38 (24.5)	72 (33.5)		1.550 (0.976–2.462)	0.063
G allele					
No	2 (1.3)	12 (5.6)	0.050	reference	–
Yes	153 (98.7)	203 (94.4)		0.221 (0.049–1.000)	0.050
rs35874116 *TAS1R2* Ile191Val					
CC	9 (5.8)	21 (9.8)	0.131	2.019 (0.880–4.632)	0.097
CT	56 (36.1)	90 (41.9)		1.391 (0.898–2.153)	0.139
TT	90 (58.1)	104 (48.4)		reference	–
C allele					
No	90 (58.1)	104 (48.4)	0.073	reference	–
Yes	65 (41.9)	111 (51.6)		1.478 (0.975–2.241)	0.066
T allele					
No	9 (5.8)	21 (9.8)	0.183	reference	–
Yes	146 (94.2)	194 (90.2)		0.569 (0.253–1.280)	0.173
Allele-based analysis	(n = 310)	(n = 430)			
rs5400 *GLUT2* Thr110Ile					
A allele	40 (12.9)	84 (19.5)	0.021	1.639 (1.089–2.466)	0.018
G allele	270 (87.1)	346 (80.5)		reference	–
rs35874116 *TAS1R2* Ile191Val					
C allele	74 (23.9)	132 (30.7)	0.046	1.413 (1.014–1.969)	0.041
T allele	236 (76.1)	298 (69.3)		reference	–

b Relationship between fillings and genes

	Endpoint[1]		p[2]	OR (95% CI)[3]	p[3]
	DMFT = 0	filling: yes			
Patient-based analysis	(n = 155)	(n = 424)			
Gender					
Boys	82 (52.9)	209 (49.3)	0.452	reference	–
Girls	73 (47.1)	215 (50.7)		1.172 (0.810–1.695)	0.401
rs5400 *GLUT2* Thr110Ile					
AA	2 (1.3)	16 (3.8)	0.271	3.099 (0.702–13.689)	0.136
AG	36 (23.2)	106 (25.0)		1.173 (0.757–1.818)	0.474
GG	117 (75.5)	302 (71.2)		reference	–
A allele					
No	117 (75.5)	302 (71.2)	0.292	reference	–
Yes	38 (24.5)	122 (28.8)		1.277 (0.835–1.954)	0.259

Downloaded by:
Masaryk University
195.113.65.1 - 10/20/2015 8:27:22 AM

Table 3. (continued)

	Endpoint[1]		p[2]	OR (95% CI)[3]	p[3]
	DMFT = 0	filling: yes			
G allele					
No	2 (1.3)	16 (3.8)	0.177	reference	–
Yes	153 (98.7)	408 (96.2)		0.336 (0.076–1.476)	0.149
rs35874116 *TAS1R2* Ile191Val					
CC	9 (5.8)	37 (8.7)	0.342	1.671 (0.774–3.605)	0.191
CT	56 (36.1)	168 (39.6)		1.219 (0.825–1.801)	0.319
TT	90 (58.1)	219 (51.7)		reference	–
C allele					
No	90 (58.1)	219 (51.7)	0.220	reference	
Yes	65 (41.9)	205 (48.3)		1.282 (0.883–1.860)	0.191
T allele					
No	9 (5.8)	37 (8.7)	0.300	reference	–
Yes	146 (94.2)	387 (91.3)		0.649 (0.306–1.379)	0.261
Allele-based analysis	(n = 310)	(n = 848)			
rs5400 *GLUT2* Thr110Ile					
A allele	40 (12.9)	138 (16.3)	0.140	1.341 (0.915–1.965)	0.133
G allele	270 (87.1)	710 (83.7)		reference	–
rs35874116 *TAS1R2* Ile191Val					
C allele	74 (23.9)	242 (28.5)	0.136	1.263 (0.935–1.706)	0.129
T allele	236 (76.1)	606 (71.5)		reference	–

c Relationship between DMFT and genes

	Endpoint[1]		p[2]	OR (95% CI)[3]	p[3]
	DMFT = 0	DMFT ≥ 1			
Patient-based analysis	(n = 155)	(n = 482)			
Gender					
Boys	82 (52.9)	242 (50.2)	0.580	reference	–
Girls	73 (47.1)	240 (49.8)		1.114 (0.775–1.601)	0.559
rs5400 *GLUT2* Thr110Ile					
AA	2 (1.3)	19 (3.9)	0.242	3.241 (0.744–14.123)	0.117
AG	36 (23.2)	120 (24.9)		1.137 (0.742–1.744)	0.556
GG	117 (75.5)	343 (71.2)		reference	–
A allele					
No	117 (75.5)	343 (71.2)	0.354	reference	–
Yes	38 (24.5)	139 (28.8)		1.248 (0.823–1.891)	0.297
G allele					
No	2 (1.3)	19 (3.9)	0.126	reference	–
Yes	153 (98.7)	463 (96.1)		0.319 (0.073–1.383)	0.127
rs35874116 *TAS1R2* Ile191Val					
CC	9 (5.8)	44 (9.1)	0.168	1.826 (0.857–3.891)	0.119
CT	56 (36.1)	197 (40.9)		1.314 (0.896–1.927)	0.163
TT	90 (58.1)	241 (50.0)		reference	–
C allele					
No	90 (58.1)	241 (50.0)	0.096	reference	–
Yes	65 (41.9)	241 (50.0)		1.385 (0.961–1.996)	0.081
T allele					
No	9 (5.8)	44 (9.1)	0.242	reference	–
Yes	146 (94.2)	438 (90.9)		0.614 (0.292–1.288)	0.197

Downloaded by:
Masaryk University
198.143.55.1 - 10/26/2015 6:27:22 AM

Table 3. (continued)

	Endpoint[1]		p[2]	OR (95% CI)[3]	p[3]
	DMFT = 0	DMFT ≥ 1			
Allele-based analysis	(n = 310)	(n = 964)			
rs5400 *GLUT2* Thr110Ile					
A allele	40 (12.9)	158 (16.4)	0.150	1.323 (0.911–1.922)	0.141
G allele	270 (87.1)	806 (83.6)		reference	–
rs35874116 *TAS1R2* Ile191Val					
C allele	74 (23.9)	285 (29.6)	0.059	1.339 (0.996–1.799)	0.053
T allele	236 (76.1)	679 (70.4)		reference	

Figures in parentheses are percentages.
[1] Contrast between DMFT = 0 (decayed/missing/filled teeth) and the given endpoint; described by absolute and relative frequencies.
[2] Fisher's exact test.
[3] Odds ratio (OR) and its statistical significance computed using logistic regression for contrast between DMFT = 0 and the given endpoint.

Table 4. Comparison of subject characteristics by genotype combinations

	GLUT2 Thr/Thr *TAS1R2* Val carriers resistant/resistant (n = 202)	*GLUT2* Ile carriers *TAS1R2* Val carriers risk/resistant (n = 104)	*GLUT2* Thr/Thr *TAS1R2* Ile/Ile resistant/risk (n = 258)	*GLUT2* Ile carriers *TAS1R2* Ile/Ile risk/risk (n = 73)
Caries	0.609±0.090	0.942±0.154	0.481±0.060	0.877±0.201
Filling	2.322±0.187	2.144±0.248	2.306±0.150	2.233±0.277
DMFT	2.936±0.214	3.087±0.596	2.794±0.171	3.096±0.369

Data are expressed as mean ± SEM. DMFT = Decayed/missing/filled teeth.

frequently dental caries ($p < 0.05$, OR = 1.639, 95% CI: 1.089–2.466). In children carrying the Val allele of *TAS1R2* Ile191Val polymorphism, caries also occurred significantly more frequently than in those with the common Ile allele ($p < 0.05$, OR = 1.413, 95% CI: 1.014–1.969). However, we did not find any significant differences between the individual caries experience (low, moderate, high) with regard to *GLUT2* and/or *TA12R2* polymorphisms (data not presented).

In contrast, no significant associations – but only trends to the same risk alleles/genotypes – between the *GLUT2* and/or *TAS1R2* polymorphisms and fillings (table 3b) were found. However, allele frequencies of the *TAS1R2* variant were marginally significantly different between children with DMFT = 0 and DMFT ≥1 (p = 0.053, OR = 1.339, 95% CI: 0.996–1.799, table 3c). The relationship between both polymorphisms and extraction (as the third part of the DMFT index) was not calculated due to the small number of children with this condition (n = 3).

Table 4 shows the means of caries, fillings and the DMFT score in the combined genotypes [Kulkarni et al., 2013]. The mean value of caries was slightly higher in the risk/risk group when compared with the resistant/resistant group; however, the trend was not statistically significant. One of the risk/resistant group even had the highest mean of caries and, in contrast, the resistant/risk group had the lowest mean value of caries from all subgroups. Similarly, no significant differences were found for fillings and DMFT indexes among these subgroups (table 4).

Discussion

Dental caries is one of the major oral health problems in most industrialized countries. It involves a complex interplay between environmental and host-related factors. Recently, genome-wide and association studies have been conducted to identify candidate regions and genes

Downloaded by: Masaryk University 198.143.56.1 - 10/20/2016 8:27:22 AM

related to caries [Werneck et al., 2010; Wang et al., 2012; Shaffer et al., 2013; Zeng et al., 2014]. In this study, we evaluated the *GLUT2* and *TAS1R2* gene variants in a relatively large group of children (n = 637) of Czech origin with and without dental caries. Till now, only one study by Kulkarni et al. [2013] investigated both genes in 80 Caucasian adults aged 21–32 years with dental caries and the authors suggested that the *GLUT2* and *TAS1R2* genotypes individually and in combination were associated with caries risk. Carriers of the Ile allele of *GLUT2* had significantly higher DMFT scores and carriers of the Val allele of *TAS1R2* had significantly lower DMFT scores in a Canadian population, and caries scores of the combined risk/risk group exceeded the mean caries scores of the single at risk genotypes alone. The disparity in caries scores was greater when the risk/risk group was compared to the resistant/resistant group [Kulkarni et al., 2013]. We found that the Ile allele of rs5400 *GLUT2 (SLC2A2)* and the Val allele of the rs35874116 *TAS1R2* genes were associated with an increased risk of caries, but not with different (low, moderate, high) caries experience, and marginally also with the DMFT index in Czech children. As the DMFT index is composed of several components, we tried to analyze it not only as a whole but per the individual parts. Although a statistically significant value was found only for caries, a trend to a higher occurrence of the same alleles/genotypes was also recorded in the 'filling component'. However, in contrast to the previous study, no significant interaction between both variants was found in our population.

Previous findings suggested that the *GLUT2* gene was involved in the regulation of postprandial glucose levels through glucose-induced insulin secretion [Mueckler et al., 1994]. Association of this *GLUT2* polymorphism with a significantly higher habitual intake of sugars was described in overweight/obese subjects and adults with diabetes mellitus type 2 [Eny et al., 2008, 2010]. Therefore, our results confirmed findings that reduced function of the *GLUT2* gene in Ile allele carriers, which results in higher daily sugar intake [Eny et al., 2008, 2010], may contribute to the increased caries occurrence in Czech children in our study. The *TAS1R2* variant is presumed to contribute to sweet taste sensitivity. Eny et al. [2008] reported lower daily sugar consumption in overweight subjects with the Val allele *TAS1R2* polymorphism, but not in lean individuals. In the present study, in contrast to the study by Kulkarni et al. [2013], we observed higher caries scores in carriers of the Val allele. This unexpected discrepancy could not be explained because we did not have dietary records regarding sugar intake.

The strength of the present study includes a relatively large sample size (n = 637), which comprised 155 caries-free and 482 caries-affected children, whereas the study by Kulkarni et al. [2013] was carried out in a small group comprising 80 young adults. In addition, we have data about the plaque index, gingival index, and calculus index indicating good oral hygiene of this cohort. In all children, the mean values of the plaque index, gingival index, and calculus index were 0.65, 0.20, and 0.67, respectively [Kukletova et al., 2012]. However, several limitations should be discussed. Firstly, we have no information regarding dietary records about the frequency of sugar/starch exposure and other existing caries risk factors, e.g. salivary test results (flow rate, bacterial scores). Secondly, the case-control design carries the risk of providing false-positive results, especially when cases and controls are from different population strata. However, all our subjects were from the same ELSPAC cohort. Thirdly, as the study participants were derived from comprehensive checkup examinees, they may be healthier compared with the general population.

In conclusion, our study implies a possible influence of specific genetic variants in the *GLUT2* and *TAS1R2* genes on caries risk also in Czech children. However, these findings require further confirmation in people with different ethnic backgrounds and assessment of other important caries risk factors. Understanding dental caries at the molecular level has the potential to facilitate new, more targeted approaches to the prevention and treatment of this common disease.

Acknowledgments

The study was supported by grant GACR GB14-37368G, IGA NT11405/6 and by project MUNI/A/1359/2014.

Author Contributions

M.K., P.B.L., L.K. and L.I.H. designed the study and drafted the paper. K.M., M.B. and M.K. performed the clinical analysis and collected the blood samples. S.L., J.K. and P.B.L. carried out the molecular analysis. L.D. and L.I.H. performed statistical analysis. All authors revised the final version of the manuscript.

Disclosure Statement

The authors declare no conflict of interest.

Downloaded by:
Masaryk University
198.143.52.1 - 10/26/2015 9:27:22 AM

References

Deeley K, Letra A, Rose EK, Brandon CA, Resick JM, Marazita ML, Vieira AR: Possible association of amelogenin to high caries experience in a Guatemalan-Mayan population. Caries Res 2008;42:8–13.

Eny KM, Wolever TM, Corey PN, El-Sohemy A: Genetic variation in *TAS1R2* (Ile191Val) is associated with consumption of sugars in overweight and obese individuals in 2 distinct populations. Am J Clin Nutr 2010;92:1501–1510.

Eny KM, Wolever TM, Fontaine-Bisson B, El-Sohemy A: Genetic variant in the glucose transporter type 2 is associated with higher intakes of sugars in two distinct populations. Physiol Genomics 2008;33:355–360.

European Longitudinal Study of Pregnancy and Childhood (ELSPAC). Paediatr Perinat Epidemiol 1989;3:460–469.

Holla LI, Musilova K, Vokurka J, Klapusova L, Pantuckova P, Kukletova M, Kukla L, Znojil V: The association of interleukin-6 (IL-6) haplotypes with plaque-induced gingivitis in children. Acta Odontol Scand 2008;66:105–112.

Kang SW, Yoon I, Lee HW, Cho J: Association between *AMELX* polymorphisms and dental caries in Koreans. Oral Dis 2011;17:399–406.

Kim UK, Wooding S, Riaz N, Jorde LB, Drayna D: Variation in the human *TAS1R* taste receptor genes. Chem Senses 2006;31:599–611.

Kukla L, Bouchalova M: European longitudinal study of pregnancy and childhood (ELSPAC) – Výzkumný projekt WHO/EURO – československá účast (in Czech). Čs Hygiena 1992;37:367–369.

Kukletova M, Izakovicova Holla L, Musilova K, Broukal Z, Kukla L: Relationship between gingivitis severity, caries experience and orthodontic anomalies in 13- to 15-year-old adolescents in Brno, Czech Republic. Community Dent Health 2012;29:179–183.

Kulkarni GV, Chng Z, Eny KM, Nielsen D, Wessman C, El-Sohemy A: Association of *GLUT2* and *TAS1R2* genotypes with risk for dental caries. Caries Res 2013;47:219–225.

Liao J, Schultz PG: Three sweet receptor genes are clustered in human chromosome 1. Mamm Genome 2003;14:291–301.

Mueckler M, Kruse M, Strube M, Riggs AC, Chiu KC, Permutt MA: A mutation in the Glut2 glucose transporter gene of a diabetic patient abolishes transport activity. J Biol Chem 1994;269:17765–17767.

Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba NJ, Zuker CS: Mammalian sweet taste receptors. Cell 2001;106:381–390.

Patir A, Seymen F, Yildirim M, Deeley K, Cooper ME, Marazita ML, Vieira AR: Enamel formation genes are associated with high caries experience in Turkish children. Caries Res 2008;42:394–400.

Roncero I, Alvarez E, Chowen JA, Sanz C, Rabano A, Vazquez P, Blazquez E: Expression of glucose transporter isoform GLUT-2 and glucokinase genes in human brain. J Neurochem 2004;88:1203–1210.

Shaffer JR, Feingold E, Wang X, Lee M, Tcuenco K, Weeks DE, Weyant RJ, Crout R, McNeil DW, Marazita ML: GWAS of dental caries patterns in the permanent dentition. J Dent Res 2013;92:38–44.

Slayton RL, Cooper ME, Marazita ML: Tuftelin, mutans streptococci, and dental caries susceptibility. J Dent Res 2005;84:711–714.

Tannure PN, Küchler EC, Falagan-Lotsch P, Amorim LM, Raggio Luiz R, Costa MC, Vieira AR, Granjeiro JM: *MMP13* polymorphism decreases risk for dental caries. Caries Res 2012a;46:401–407.

Tannure PN, Küchler EC, Lips A, Costa Mde C, Luiz RR, Granjeiro JM, Vieira AR: Genetic variation in *MMP20* contributes to higher caries experience. J Dent 2012b;40:381–386.

Volckova M, Borilova Linhartova P, Trefna T, Vlazny J, Musilova K, Kukletova M, Kukla L, Izakovicova Holla L: Lack of association between lactotransferrin polymorphism and dental caries. Caries Res 2014;48:39–44.

Wang X, Shaffer JR, Weyant RJ, Cuenco KT, DeSensi RS, Crout R, McNeil DW, Marazita ML: Genes and their effects on dental caries may differ between primary and permanent dentitions. Caries Res 2010;44:277–284.

Wang X, Shaffer JR, Zeng Z, Begum F, Vieira AR, Noel J, Anjomshoaa I, Cuenco KT, Lee MK, Beck J, Boerwinkle E, Cornelis MC, Hu FB, Crosslin DR, Laurie CC, Nelson SC, Doheny KF, Pugh EW, Polk DE, Weyant RJ, Crout R, McNeil DW, Weeks DE, Feingold E, Marazita ML: Genome-wide association scan of dental caries in the permanent dentition. BMC Oral Health 2012;12:57.

Werneck RI, Mira MT, Trevilatto PC: A critical review: an overview of genetic influence on dental caries. Oral Dis 2010;16:613–623.

WHO: Oral Health Surveys: Basic Methods, ed 4. Geneva, WHO, 1997.

Zeng Z, Feingold E, Wang X, Weeks DE, Lee M, Cuenco DT, Broffitt B, Weyant RJ, Crout R, McNeil DW, Levy SM, Marazita ML, Shaffer JR: Genome-wide association study of primary dentition pit-and-fissure and smooth surface caries. Caries Res 2014;48:330–338.

Downloaded by:
Masaryk University

Attachment 11 Article "Influence of diabetes mellitus on dental condition and periodontal status"

Původní práce
Original Article

Vliv onemocnění diabetes mellitus na stav chrupu a parodontu

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 1,
s. 3–12

(Původní práce – klinická a mikrobiologická studie)

Influence of Diabetes Mellitus on Dental Condition and Periodontal Status

(Original Article – Clinical and Microbial Study)

Poskerová H.[1], Bořilová Linhartová P.[1,2], Cvanová M.[3], Izakovičová Holá L.[1,2]

[1]Stomatologická klinika LF MU a FN u sv. Anny, Brno
[2]Ústav patologické fyziologie LF MU, Brno
[3]Institut biostatistiky a analýz LF MU, Brno

SOUHRN

Úvod a cíl: Diabetes mellitus (DM) a parodontitida jsou chronická zánětlivá onemocnění s vysokou prevalencí, která mají některé společné znaky a mohou se vzájemně ovlivňovat. Cílem studie bylo posoudit stav chrupu a parodontu u českých pacientů s DM a přispět k poznání rizikových faktorů obou chorob.
Metody: Vyšetřili jsme a vzájemně porovnali tři skupiny osob: diabetiky 1. typu (T1DM), diabetiky 2. typu (T2DM) a osoby bez diabetu (kontroly). Účastníky studie jsme vyšetřili klinicky, rentgenologicky a dále jsme se zaměřili na analýzu mikrobiálního složení subgingiválního plaku. Hodnocení výsledků jsme provedli s využitím statistického softwaru IBM SPSS Statistics 23.
Výsledky: Do studie jsme zahrnuli celkem 141 osob (32 s T1DM, 51 s T2DM, 58 kontrol) ve věku 35–65 let vyšetřených během let 2010–2015. Zjistili jsme, že stav chrupu a parodontu u diabetiků, a to především u T2DM, byl horší než u kontrol. Tento nález se týkal plak indexu a gingiválního indexu, počtu extrahovaných zubů, přítomnosti zubních náhrad a dalších parametrů hodnotících přítomnost a závažnost parodontitidy. Rozdíly v zastoupení parodontálních patogenů a jejich množství mezi skupinami byly minimální. Pacienti s T2DM a diabetickou nefropatií měli horší stav parodontu; toto se nám nepodařilo prokázat u pacientů s nedostatečně kompenzovaným diabetem, resp. delší dobou trvání choroby.
Závěr: U diabetiků lze předpokládat častější výskyt zánětlivých parodontopatií i dalších onemocnění dutiny ústní. Z klinického vyšetření a anamnestických údajů může zubní lékař vyslovit podezření na DM a včasnou léčbou parodontitidy pak přispět ke zlepšení celkového zdravotního stavu pacienta.
Klíčová slova: ***diabetes mellitus – chronická parodontitida – parodontologický index – rentgenologické vyšetření – parodontální patogeny***

SUMMARY

Introduction, aim: Diabetes mellitus (DM) and periodontitis are chronic inflammatory diseases with high prevalence. Both conditions possess some common traits and can mutually affect each other. The aim of the study was to investigate the state of teeth and periodontium in Czech diabetic patients and contribute to the identification of genetic risk factors of both diseases.
Methods: We examined and mutually compared three groups of subjects: type 1 diabetic patients (T1DM), type 2 diabetic patients (T2DM) and subjects without diabetes mellitus (controls). The subjects included in the study underwent clinical, radiological and further we focused on the analysis of microbiological composition of subgingival plaque. Evaluation of results was performed by statistical software IBM SPSS Statistics 23.
Results: The study comprised a total of 141 subjects (32 with T1DM, 51 with T2DM, 58 controls), aged 35–65 years, examined during 2010–2015. We found that the state of teeth and periodontium in diabetic

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 1,
s. 3–12

(mainly T2DM) patients was worse than in subjects without diabetes. This finding was confirmed for plaque index and gingival index, number of extracted teeth, the presence of teeth replacement and other parameters evaluating the presence and severity of periodontitis. The differences in the representation of periodontal pathogens and their quantity among the groups were minimal. Patients with T2DM and diabetic nephropathy had worse state of the periodontium; this finding was not proven in patients with insufficiently controlled diabetes or the disease duration.
Conclusion: In diabetic patients, a more frequent occurrence of inflammatory periodontal diseases and other diseases of the oral cavity is expected. Based on clinical examination and anamnestic data, the dentist can presume DM, early treatment of periodontal disease contributes to improved general health of the patient.
Keywords*: diabetes mellitus – chronic periodontitis – periodontal index – radiological examination – periodontal pathogens*

Čes. Stomat., roč. 118, 2018, č. 1, s. 3–12

ÚVOD

Vliv onemocnění diabetes mellitus na stav dutiny ústní, zejména na tkáně parodontu, tvrdé zubní tkáně, slinné žlázy a ústní sliznice je znám a sledován již několik desetiletí. Řada studií potvrzuje tuto souvislost zejména u osob s dlouhodobě přítomným a nedostatečně kompenzovaným onemocněním [1, 6, 14, 29]. Za hlavní etiologický faktor vzniku a rozvoje parodontitidy u diabetiků je považována dlouhodobě zvýšená hladina glukózy v krvi vedoucí ke vzniku pozdních produktů glykace. Tyto pak, spolu s dalšími patogenetickými mechanismy, způsobují strukturální a funkční změny kapilár, poruchy metabolismu kolagenu, změny funkce imunitních buněk a poruchy kostního metabolismu [37]. V poslední době se výzkum zaměřuje na objasnění souvislostí mezi diabetem a parodontitidou na molekulární a buněčné úrovni. Ukazuje se, že vztah mezi oběma chorobami je oboustranný. Diabetes je rizikovým faktorem pro rozvoj zánětlivých onemocnění parodontu, pokročilá parodontitida může vlivem zvýšené produkce prozánětlivých mediátorů přispět například ke vzniku inzulinové rezistence [8]. Předpokládá se, že léčba parodontitidy by mohla zlepšit kompenzaci DM [16]. Tato práce si klade za cíl porovnat stav chrupu a parodontu u českých pacientů s diabetem a bez diabetu a přispět k poznání rizikových faktorů obou chorob.

METODIKA

Sledovaný soubor tvořilo celkem 141 osob (53 mužů, 88 žen). Vyšetřili jsme a vzájemně porovnali tři skupiny: diabetiky 1. typu (T1DM), diabetiky 2. typu (T2DM) a osoby bez diabetu (kontroly) (tab. 1). Vyšetřování jsme prováděli v letech 2010–2015, všichni pacienti byli seznámeni s jeho průběhem a svou účast ve studii potvrdili podpisem informovaného souhlasu. Studie byla schválena etickou komisí Lékařské fakulty Masarykovy univerzity a Fakultní nemocnice u svaté Anny v Brně. Kritéria pro zařazení do studie byla následující: více než 12 vlastních zubů (kromě třetích molárů) a u diabetiků délka trvání diabetu od jeho diagnostiky více než dva roky. Vyloučili jsme účast těhotných a kojících žen, pacientů po transplantacích orgánů, osob se závažnými systémovými chorobami a jedinců dlouhodobě užívajících kortikosteroidy, imunosupresiva a nesteroidní antiflogistika. Do studie také nebyli zařazeni pacienti, kteří podstoupili parodontologickou léčbu během uplynulých šesti měsíců a kteří užívali v posledních třech měsících antibiotika.

Anamnéza, klinické a rentgenologické vyšetření

S každým pacientem zařazeným do studie jsme vyplnili podrobnou anamnézu včetně indexu tělesné hmotnosti (BMI). U diabetiků jsme zaznamenali dobu trvání a typ diabetu, jeho léčbu a přítomnost komplikací. Ve spolupráci s ošetřujícími lékaři jsme zjišťovali hodnotu glykovaného hemoglobinu (HbA1c), z níž lze odvodit stupeň kompenzace diabetu [18]. U zdravých osob jsme vyloučili přítomnost nerozpoznaného diabetu vyšetřením glykémie z kapilární krve nalačno za použití glukometru.

Účastníci studie dále podstoupili komplexní stomatologické vyšetření včetně parodontologického (nehodnotili jsme stav třetích molárů). Zaznamenávali jsme počet přítomných zubů, index kazivosti chrupu (KPE) a přítomnost zubních náhrad. V rámci parodontologického vyšetření jsme stanovili hodnoty plak indexu (PI) a gingiválního indexu (GI) [28], dále jsme u každého zubu zjišťovali

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 1,
s. 3–12

hloubku parodontálních chobotů (HPCH) na čtyřech ploškách. Ve stejných lokalitách jsme změřili velikost gingiválních recesů (GR) a zapsali jsme i místa s výraznou hyperplazií gingivy. Z těchto údajů jsme následně vypočítali klinickou ztrátu úponu (CAL). Podkladem pro posouzení resorpce alveolární kosti bylo rentgenologické vyšetření. Do souboru byli zařazeni pouze pacienti s chronickou parodontitidou, kterou jsme vyhodnotili podle závažnosti a rozdělili do tří skupin s ohledem na CAL. Následující podmínky platily vždy pro alespoň čtyři různé lokality [41]:

NP:	bez parodontitidy
P1: CAL 1–2 mm	parodontitida mírná
P2: CAL 3–4 mm	parodontitida středně pokročilá
P3: CAL 5 a více mm	parodontitida pokročilá

Vyšetření parodontálních patogenů

U všech pacientů jsme dále nechali stanovit sedm vybraných parodontálních patogenů (*Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Treponema denticola*, *Parvimonas micra*, *Fusobacterium nucleatum*) testem StomaGene® (Protean, České Budějovice). Pomocí tohoto semikvantitativního testu lze také určit míru rizika resorpce závěsného aparátu.

Statistické hodnocení

Ke statistickému hodnocení byl použit Kruskalův-Wallisův a Mannův-Whitneyho test pro srovnání rozdělení pravděpodobnosti spojitých proměnných ve skupinách. Fisherovým exaktním testem byly hodnoceny závislosti dvou kategoriálních proměnných. Za hladinu statistické významnosti byla zvolena P-hodnota ≤ 0,05. Výsledky porovnání jednotlivých kategorií (T2DM vs. kontroly, T1DM vs. kontroly, T2DM+T1DM vs. kontroly) byly korigovány na mnohonásobné testování pomocí Bonferroniho korekce. I přes určité věkové rozdíly ve vyšetřovaných skupinách je možné porovnávat T2DM a T1DM se

Tab. 1 Charakteristika souboru a klinické parametry (n = 141)

Parametr **medián (min; max)**	**Kontroly (n = 58)**	**T1DM (n = 32)**	**T2DM (n = 51)**
Muži (n = 53) **Ženy** (n = 88)	13 (22,4 %) 45 (77,6 %)	12 (37,5 %) 20 (62,5 %)	28 (54,9 %) 23 (45,1 %)
Věk [průměr ± SD v letech]	52,0 ± 9,3	49,4 ± 7,8	55,9 ± 7,2
Kuřáci (n = 29) **Nekuřáci** (n = 112)	10 (17,2 %) 48 (82,8 %)	8 (25,0 %) 24 (75,0 %)	11 (21,6 %) 40 (78,4 %)
BMI [kg/m²]	25,9 (19,5; 37,1)	24,8 (21,8; 31,6)	31,3 (20,9; 52,9)
KPE	0,5 (0,1; 1,0)	0,5 (0,1; 0,9)	0,5 (0,1; 0,9)
Extrahované zuby	1,0§§ (0,0; 10,0)	1,0 (0,0; 10,0)	3,0*** (0,0; 16,0)
Zubní náhrady (n = 82)	27 (46,6 %)	18 (56,3 %)	37 (72,5 %)*
PI	0,6§§§ (0,1; 2,1)	1,0* (0,3; 2,1)	1,2*** (0,1; 2,3)
GI	0,9§§ (0,0; 1,7)	1,0 (0,4; 1,8)	1,1** (0,3; 2,4)
HPCH [mm]	3,0§§§ (1,7; 6,0)	3,3* (2,6; 5,8)	3,7*** (2,1; 7,3)
CAL [mm]	3,6§§§ (1,9; 7,0)	4,1* (2,7; 6,9)	4,7*** (2,1; 9,6)
Kostní resorpce dle rtg snímku více než 2/3 délky kořene	10 (17,2 %)§§	13 (40,6 %)	21 (41,2 %)**

* P<0,05 v porovnání ke kontrolám, ** P<0,01 v porovnání ke kontrolám, *** P<0,001 v porovnání ke kontrolám (po Bonferroniho korekci)
# P<0,05 v porovnání k T1DM , ## P<0,01 v porovnání k T1DM (po Bonferroniho korekci)
§ P<0,05 kontroly vs. T1DM+T2DM, §§ P<0,01 kontroly vs. T1DM+T2DM, §§§ P<0,001 kontroly vs. T1DM+T2DM (po Bonferroniho korekci)
BMI = body mass index, CAL = klinická ztráta úponu, GI = gingivální index, HPCH = hloubka parodontálního chobotu, KPE = kaz/plomba/extrahovaný zub index, n = počet, PI = plak index, SD = směrodatná odchylka, T1DM = diabetici 1. typu, T2DM = diabetici 2.typu

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 1,
s. 3–12

skupinou osob bez diabetu každou zvlášť, i pokud diabetiky sloučíme v jeden celek.

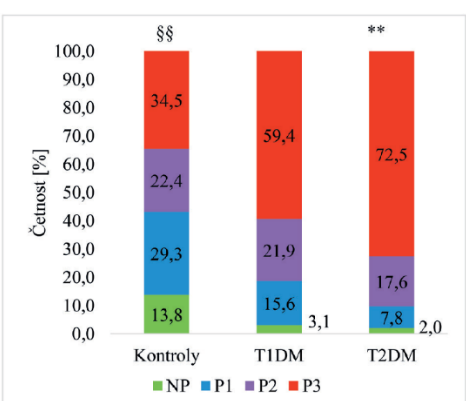
VÝSLEDKY

Porovnání úrovně ústní hygieny, zánětu gingivy a stavu chrupu u osob s diabetem a kontrol

Z průměrných hodnot nebo mediánu PI a GI lze odvodit, že diabetici v našem souboru měli ve srovnání s osobami bez diabetu horší ústní hygienu a častěji přítomnou gingivitidu. Statisticky nevýznamné rozdíly byly zjištěny pouze při porovnání intenzity zánětu dásní mezi skupinami T1DM a kontrol (tab. 1). U indexu KPE jsme mezi sledovanými skupinami významný rozdíl v hodnotách nezjistili. Diabetici 2. typu však měli vyšší počet extrahovaných zubů než osoby bez diabetu. Také zubní náhrady byly častěji přítomny u osob s T2DM, a to zejména u žen (78,3 % diabetiček 2. typu vs. 44,4 % žen bez diabetu). U mužů tato souvislost nebyla statisticky významná. U diabetiků 1. typu jsme hodnoty KPE ani přítomnost náhrad neprokázali signifikantně odlišné od kontrol (tab. 1).

Porovnání prevalence a závažnosti parodontitidy u osob s diabetem a kontrol

U diabetiků jsme ve srovnání s kontrolami našli hlubší parodontální choboty, větší ztrátu úponu, významnější resorpci alveolární kosti a také častější výskyt pokročilé parodontitidy. Tyto odchylky jsme zaznamenali zejména při porovnávání skupin T2DM a kontrol (tab. 1, graf 1). Při srovnání skupin T1DM a kontrol byl významný rozdíl pouze u parametrů HPCH a CAL (tab. 1).



Graf 1 Porovnání prevalence a závažnosti parodontitidy u osob s diabetem a u kontrolní skupiny
NP = bez parodontitidy, P1/2/3 = mírná/středně pokročilá/pokročilá parodontitida, T1DM = diabetici 1. typu, T2DM = diabetici 2. typu
** P<0,01 v porovnání ke kontrolám (po Bonferroniho korekci)
§§ P<0,01 kontroly vs. T1DM+T2DM (po Bonferroniho korekci)

Vliv kouření na stav chrupu a parodontu u osob s diabetem a kontrol

V naší studii bylo celkem 20,6 % kuřáků. Předpoklad, že by tito kuřáci měli vyšší index plaku a nižší gingivální index ve srovnání s nekuřáky byl patrný pouze u některých podskupin, výsledky však nebyly statisticky významné. Po analýze porovnávající index KPE a stav parodontu mezi kuřáky a nekuřáky jsme zjistili významný rozdíl jen u diabetiků 2. typu u parametru ztráta úponu. CAL byl u kuřáků (nad 5 i nad 10 cigaret za den) s T2DM větší než u celoživotních nekuřáků s T2DM (tab. 2).

Vliv nadváhy a obezity na stav chrupu a parodontu u osob s diabetem a kontrol

V našem souboru se potvrdil všeobecný předpoklad, že nejvíc obézních bude mezi diabetiky 2. typu (60,8 %), obézní diabetik 1. typu byl pouze jeden a osob bez diabetu s obezitou bylo 22,4 %. Po statistickém zhodnocení výsledků se ukázalo, že obézní a osoby s nadváhou z kontrolní skupiny (a to zejména ženy) měly významně vyšší hodnotu plak indexu a obézní pak vyšší hodnotu gingiválního indexu ve srovnání s osobami s normální váhou (tab. 3a). U diabetiků jsme odlišné hodnoty plak indexu prokázali pouze u žen s T2DM. U obézních diabetiček 2. typu jsme dále zjistili hlubší sondáž parodontálních chobotů (tab. 3b). Nepotvrdili jsme však předpoklad, že obézní osoby trpí častěji pokročilou parodontitidou.

Souvislost mezi kompenzací diabetu a stavem parodontu u diabetiků

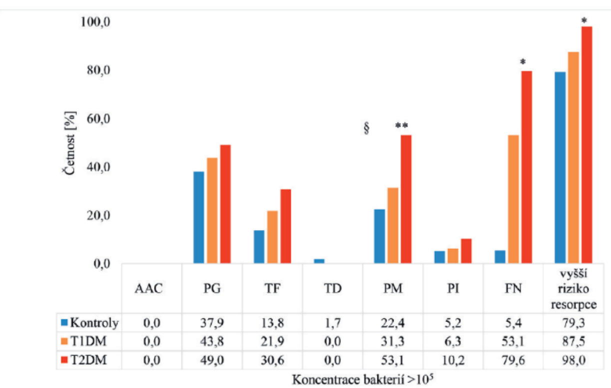
Ve sledovaném souboru diabetiků se nám nepodařilo prokázat u pacientů s nedostatečnou kompenzací horší stav parodontu (P3) než u těch dobře kompenzovaných. Tento výsledek je pravděpodobně ovlivněn nerovnoměrným rozdělením osob v jednotlivých skupinách. Například 93,8 % diabetiků 1. typu patřilo mezi neuspokojivě kompenzované (u osob s T2DM to bylo 74,5 %). Mezi diabetiky se zdravým parodontem (NP) byl pouze jeden s T1DM a jeden s T2DM (oba s neuspokojivou kompenzací).

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 1,
s. 3–12

Tab. 2 Vliv kouření na stav chrupu a parodontu u osob s T2DM (n = 51)

Parametr medián (min; max)	**Celoživotní nekuřáci (n = 33)**	**Kuřáci nad 5 cig/den (n = 10)**	**Kuřáci nad 10 cig/den (n = 8)**
KPE	0,5 (0,1; 0,9)	0,6 (0,2; 0,9)	0,6 (0,2; 0,9)
PI	1,2 (0,1; 2,3)	1,1 (0,4; 2,1)	0,9 (0,4; 2,1)
GI	1,1 (0,3; 2,4)	1 (0,5; 1,5)	1 (0,5; 1,1)
HPCH [mm]	3,4 (2,1; 7,3)	4,1 (2,4; 5,7)	4,1 (2,4; 5,7)
CAL [mm]	4,2 (2,1; 7,4)	5,4* (2,8; 8,7)	5,4* (2,8; 8,7)
P3	20 (60,6 %)	9 (90,0 %)	7 (87,5 %)

* P<0,05 v porovnání k nekuřákům (po Bonferroniho korekci)
CAL = klinická ztráta úponu, cig = cigareta, GI = gingivální index, HPCH = hloubka parodontálního chobotu, KPE = kaz/plomba/extrahovaný zub index, n = počet, P3 = pokročilá parodontitida, PI = plak index, SD = směrodatná odchylka, T2DM = diabetes mellitus 2 . typu



Graf 2 Porovnání subgingivální mikroflóry a rizika resorpce alveolární kosti u osob s diabetem a u kontrolní skupiny

AAC = *Aggregatibacter actinomycetemcomitans*,
FN = *Fusobacterium nucleatum*, PG = *Porphyromonas gingivalis*, PI = *Prevotella intermedia*,
PM = *Parvimonas micra*, T1DM = diabetici 1. typu, T2DM = diabetici 2. typu,
TD = *Treponema denticola*, TN = *Tannarella forsythia*
* P<0,05 v porovnání ke kontrolám, ** P<0,01 v porovnání ke kontrolám (po Bonferroniho korekci)
§ P<0,05 kontroly vs. T1DM+T2DM (po Bonferroniho korekci)

Souvislost mezi délkou trvání diabetu a stavem parodontu u diabetiků

Pacienti s T1DM měli diabetes průměrně 26,4 roku (medián 24,0 roků), u T2DM byla průměrná doba jeho trvání 9,5 roku (medián byl 7,0 let). Nepodařilo se nám prokázat, že by pacienti s dlouhodobě přítomným diabetem měli statisticky významně horší stav parodontu (P3) než ti s krátkou dobou jeho vlivu. Tento výsledek může být opět ovlivněn nerovnoměrným rozdělením osob v jednotlivých kategoriích. Například pouze jeden diabetik 1. typu měl diabetes přítomný méně než deset let (byl zařazen do skupiny P1).

Souvislost mezi přítomností diabetických komplikací a stavem parodontu u diabetiků

Ze sledovaných diabetických komplikací (nefropatie, retinopatie a neuropatie) se nám podařilo prokázat statisticky významný vztah pouze mezi parodontitidou a nefropatiemi u diabetiků 2. typu. Zjistili jsme, že postižení ledvin měli jen ti s pokročilou parodontitidou, zatímco osoby ve skupinách NP, P1, P2 touto komplikací netrpěly.

Porovnání subgingivální mikroflóry a rizika resorpce alveolární kosti u osob s diabetem a kontrol

Při porovnání výskytu vybraných bakterií (parodontálních patogenů) jsme pozorovali rozdíl v jejich zastoupení pouze ve skupině T2DM oproti kontrolám. Statisticky významné změny jsme však zjistili

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 1,
s. 3–12

Tab. 3a Vliv nadváhy a obezity na stav parodontu u žen z kontrolní skupiny (n=45)

Parametr medián (min; max)	BMI < 25	BMI ≥ 25	BMI ≥ 30
PI	0,4 (0,1; 2,1)	0,9 (0,2; 1,7)	0,9* (0,1; 1,7)
GI	0,6 (0,1; 1,7)	1,1* (0,0; 1,6)	1,0 (0,0; 1,6)

Tab. 3b Vliv nadváhy a obezity na stav parodontu u žen s T2DM (n= 23)

Parametr medián (min; max)	BMI < 25	BMI ≥ 25	BMI ≥ 30
PI	0,7 (0,1; 0,7)	1,3* (0,7; 2,3)	1,3* (0,3; 2,3)
HPCH [mm]	3,2 (3,1; 3,6)	4,1* (3,1; 6,1)	3,7 (2,1; 6,1)

BMI = body mass index, GI = gingivální index, HPCH = hloubka parodontálního chobotu, n = počet, PI = plak index, T2DM = diabetes mellitus 2 . typu
* P<0,05 v porovnání k BMI ≥ 30 vs. < 25 a BMI ≥ 25 vs. < 25 (po Bonferroniho korekci)

jen pro *P. micra* a *F. nucleatum*. V kategorii rizika resorpce alveolární kosti jsme zaznamenali významný rozdíl pro kostní resorpci ve skupině T2DM oproti kontrolám (graf 2).

DISKUSE

Ve světové literatuře se vztahem diabetu a parodontitidy zabývá více než dva tisíce odborných článků (původních vědeckých prací i souhrnných příspěvků) [3]. V České republice se studiu stavu chrupu a parodontu u diabetiků dlouhodobě věnuje pouze prof. MUDr. Jana Dušková, DrSc. [10–13], genetickými aspekty parodontitidy u diabetiků se pak zabývá prof. MUDr. Lydie Izakovičová Hollá, Ph.D. [4, 5]. Cílem této práce bylo přispět k širšímu poznání problematiky v ČR a porovnat výsledky získané studiem vlastního souboru pacientů se situací v ostatních zemích. Naše výsledky hodnocení stavu chrupu, parodontu a výskytu mikroorganismů v subgingiválním plaku lze v mnoha ohledech považovat za blízké literárním údajům.

Úroveň ústní hygieny, gingiválního zánětu a stavu chrupu u osob s diabetem a kontrol

Ve shodě s našimi závěry uvádějí autoři výzkumných prací, že diabetici 2. typu měli ve srovnání s osobami bez diabetu horší ústní hygienu (PI) [6, 20] a častěji se u nich vyskytovala gingivitida (GI) [24]. V naší studii měli diabetici 2. typu více extrahovaných zubů než osoby bez diabetu, což Campus a kol. potvrdili pouze u T2DM starších než padesát let [6].

Prevalence a závažnost parodontitidy u osob s diabetem a kontrol

U diabetiků jsme ve srovnání s osobami bez diabetu prokázali přítomnost hlubších parodontálních chobotů, větší ztrátu úponu, rozsáhlejší resorpci alveolární kosti a také častější výskyt pokročilé parodontitidy. Statisticky významná byla tato zjištění zejména pro diabetiky 2. typu. Podobné výsledky uvádějí i literární zdroje včetně rozsáhlých metaanalýz [9, 25].

Chávary a kol. provedli metaanalýzu 57 studií (průřezových i longitudinálních) zkoumajících vztah mezi diabetem (T2DM i T1DM) a onemocněním parodontu [9]. Z výsledků odvodili, že diabetes 2. typu je rizikovým faktorem pro vznik a rozvoj parodontitidy. U osob s diabetem 1. typu tento vztah nebyl významný, což bylo způsobeno zejména nízkým věkem pacientů s T1DM zahrnutých ve výzkumných pracích. Khader a kol. ve své metaanalýze 23 studií zjistili, že pokročilá parodontitida se vyskytovala častěji u diabetiků obou typů ve srovnání s osobami bez diabetu [25]. I ve své další práci prokázali, že diabetici 2. typu trpí vážnějším onemocněním parodontu [24]. Podobné závěry učinili i Hintao a kol., Lim a kol. a Campus a kol. [6, 20, 27]. Garcia a kol. při rozboru dat zjistili, že závažnost parodontitidy pozitivně korelovala s hladinou HbA1c [19]. Rajhans a kol. vyšetřili 1500 diabetiků, přičemž glykemický status signifikantně koreloval se závažností parodontitidy [34].

Problémem vzájemného porovnání jednotlivých studií jsou však rozdílné přístupy v metodice. Jak uvádí Holtfreter se spolupracovníky v recentním

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 1,
s. 3–12

sdělení, v současné době neexistuje univerzálně akceptovaná definice chronické parodontitidy [21]. Je sice téměř vždy založena na parametrech HPCH a CAL, ale jednotlivá doporučení pro klasifikaci se mezi studiemi liší.

Vliv kouření a obezity na stav chrupu a parodontu u osob s diabetem a kontrol

Předpoklad, že by kuřáci měli horší stav chrupu a parodontu jsme zaznamenali pouze u některých podskupin. Možná i proto, že kuřáků bylo pětkrát méně než nekuřáků. Podrobnou souhrnnou práci s názvem „Smoking and diabetes – The double health hazard!" zveřejnili Fagard a Nilsson [17]. Analyzují v ní množství odborných studií na toto téma a uvádějí, že silní kuřáci měli zvýšené riziko vzniku diabetu (téměř dvakrát ve srovnání s nekuřáky) a také diabetických mikrovaskulárních a makrovaskulárních komplikací. Syrjälä a kol. zjistili, že u kuřáků s diabetem byly hlubší parodontální choboty a větší ztráta úponu ve srovnání s nekuřáky diabetiky i s nekuřáky bez diabetu [38]. K podobným závěrům u diabetiků dospěli i Jansson a kol. [23]. Moore a kol. při studiu souboru 320 diabetiků 1. typu zjistili, že kuřáci měli pokročilou parodontitidu téměř desetkrát častěji než nekuřáci [31].

První práce, které začaly zkoumat vztahy mezi stavem parodontu a obezitou se objevily na přelomu 20. a 21. století a mnoho z nich potvrzuje předpokládanou souvislost mezi oběma chorobami [7, 22, 30, 32]. Naše výsledky ukázaly, že zejména obézní ženy měly vyšší hodnotu PI a GI. U diabetiček 2. typu jsme dále zjistili hlubší sondáž parodontálních chobotů. Chaffee se spolupracovníky provedli rozsáhlou metaanalýzu 57 nezávislých populačních studií na téma obezita a parodontitida [7]. Zjistili, že pacienti s parodontitidou měli o jednu třetinu vyšší prevalenci obezity než osoby se zdravým parodontem. U obézních pak byla zjištěna větší ztráta úponu. Vztah mezi obezitou a parodontitidou byl významnější u žen, nekuřáků a mladších osob než u běžné dospělé populace. Nishida a kol. ve své studii zabývající se podrobně vlivem kouření a obezity na výskyt parodontitidy uvádějí, že kouření je nejsilnějším vnějším rizikovým faktorem pro přítomnost parodontitidy, obezita je pak významným přídatným faktorem [33].

Vztah mezi kompenzací diabetu, délkou jeho trvání a výskytem a závažností parodontitidy u diabetiků

V našem souboru diabetiků jsme při hodnocení těchto souvislostí nezjistili statisticky významný rozdíl. Z literárních zdrojů však vyplývá, že výše zmíněný vztah řada prací potvrdila. Tsai se spolupracovníky v rozsáhlé epidemiologické studii (4343 osob ve věku vyšším než 45 let) zjistili, že nedostatečně kompenzovaní diabetici 2. typu měli 2,9krát vyšší prevalenci pokročilé parodontitidy než osoby bez diabetu [40]. Apoorva a kol. v souboru 600 osob ve věku od 35 do 75 let popsali výskyt pokročilé parodontitidy u nedostatečně kompenzovaných 2,42krát, u uspokojivě kompenzovaných 2,10krát a u výborně kompenzovaných 1,97krát častější než u osob bez diabetu [2]. Khader a kol. uvádějí, že závažnost parodontitidy byla signifikantně vyšší u pacientů s T2DM trvajícím déle než pět let [24]. Podobně i Rajhans a kol. zjistili, že délka diabetu korelovala s prevalencí a závažností parodontitidy [34]. Moore a kol. udávají tuto souvislost i pro pacienty s T1DM [31].

Diabetické komplikace a stav parodontu u diabetiků

V našem souboru se statisticky významný vztah mezi přítomností komplikací diabetu a parodontitidou podařilo prokázat pouze u diabetiků 2. typu s nefropatiemi. Shultis a kol. popsali vztah mezi stavem parodontu a diabetickými komplikacemi u diabetiků 2. typu populace Pima indiánů (529 subjektů) [36]. Makroalbuminurie a renální selhávání se u osob se středně pokročilou a pokročilou parodontitidou, i u těch již zcela bezzubých, vyskytovaly častěji (2,3krát; 3,5krát; respektive 4,9krát) než u diabetiků se zdravým nebo jen mírně postiženým parodontem. Saremi a kol. studovali také populaci Pima indiánů (628 účastníků) v prospektivní dlouhodobé studii [35]. U diabetiků s těžkou parodontitidou popsali úmrtnost na diabetickou nefropatii 8,5krát vyšší a na ischemickou chorobu srdeční 2,3krát vyšší než u diabetiků s mírně pokročilou parodontitidou nebo bez ní. Stav chrupu u pacientů s diabetickými komplikacemi hodnotila i Dušková v ČR [10]. V souboru osob zařazených do transplantačního programu pro diabetickou nefropatii a zejména ve skupině osob vyšetřených v podiatrické poradně pro syndrom diabetické nohy zjistila, že počet osob bez vlastního chrupu mnohonásobně převyšoval počet bezzubých stejně starých, ale zdravých jedinců.

Subgingivální mikroflóra u diabetiků a kontrol

Dříve se přepokládalo, že u pacientů s diabetem má podstatný vliv na stav parodontu speciální bakteriální mikroflóra, to ale následné výzkumy

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 1,
s. 3–12

nepotvrdily. Autoři, kteří se zabývali složením subgingiválního biofilmu zjistili, že mikroflóra je v této lokalitě u diabetiků obou typů a ve zdravé populaci velmi podobná [15, 20, 26, 39, 42]. Taylor a kol. popsali podrobně jedenáct studií (z let 1989–2008), ve kterých autoři analyzovali složení subgingivální flóry různými metodami (kultivace, PCR analýza, vyšetření protilátek) [39]. V závěru se shodli na tom, že není statisticky významný rozdíl ve složení mikroflóry mezi diabetiky a zdravými osobami (případně mezi diabetiky s vysokou a nízkou hladinou HbA1c), pouze některé bakterie se u pacientů s diabetem mohou vyskytovat ve vyšším počtu. V našem souboru jsme při studiu zastoupení vybraných bakterií v souladu s literaturou zjistili, že všechny sledované bakterie se vyskytovaly ve všech sledovaných skupinách (u T2DM, T1DM i u kontrol), takže žádný z patogenů nebylo možno považovat za specifický pro diabetiky. Pozorovali jsme pouze o něco vyšší kvantitativní zastoupení mikroorganismů ve skupině T2DM oproti kontrolám.

Limity naší studie

Za hlavní limitaci této práce považujeme relativně nízký počet osob, které jsme zařadili do závěrečného hodnocení. Z primárně vyšetřeného souboru 225 dospělých osob (ve věku 23–83 let) jsme vybrali menší soubor (n = 141) ve věkovém rozmezí 35–65 let tak, aby byly skupiny T1DM, T2DM a kontrol, pokud jde o věk, vzájemně porovnatelné. Po celou dobu trvání výzkumného projektu jsme se potýkali s nedostatkem zájemců, část kandidátů pak nesplňovala relativně přísné podmínky pro zařazení (např. dostatečný počet vlastních zubů, nutnost odběru krve, daná kritéria pro zdravotní stav a medikaci). Ačkoli výběr osob nebyl z naší strany ovlivněn, je velmi pravděpodobné, že odesílající lékaři doporučovali parodontologické vyšetření zejména těm pacientům, kteří si na potíže s chrupem a dásněmi stěžovali.

Přednost studie spatřujeme v tom, že veškerá klinická vyšetření prováděla pouze jedna osoba, hodnoceny byly všechny přítomné zuby a řada měření byla prováděna na několika ploškách jednotlivých zubů. Zaznamenávali jsme poměrně velký počet vybraných anamnestických, klinických a mikrobiálních parametrů, a tak jsme získali o jednotlivých pacientech dostatečné množství dat. Bylo tedy možné provést i multivariační analýzu interakcí mezi jednotlivými faktory, která by mohla přispět k lepšímu pochopení etiopatogeneze parodontitidy a/nebo diabetu.

ZÁVĚR

V klinické části studie jsme zjistili, že:

1. stav chrupu u diabetiků 2. typu byl horší než u osob kontrolní skupiny. Týkalo se to počtu extrahovaných zubů a přítomnosti zubních náhrad, nikoli však indexu KPE;
2. diabetici 2. typu měli ve srovnání s kontrolami větší množství zubního povlaku a častěji přítomnou gingivitidu;
3. diabetici ve srovnání s kontrolami vykazovali přítomnost hlubších parodontálních chobotů, větší ztrátu úponu, významnější rentgenologicky ověřenou resorpci alveolární kosti a častější výskyt pokročilé parodontitidy. Mezi skupinami T1DM a kontrol byl zjištěn statisticky významný rozdíl pouze v hloubce parodontálních chobotů a ztrátě úponu;
4. u diabetiků 2. typu s diabetickou nefropatií byla častěji přítomna pokročilá parodontitida než u pacientů bez této komplikace. Postižení ledvin bylo diagnostikováno pouze u T2DM s P3;
5. nedostatečně kompenzovaní diabetici a ti s dlouhodobě přítomným diabetem neměli v našem souboru oproti předpokladu horší stav parodontu než ostatní osoby s DM;
6. při porovnání stavu chrupu a parodontu mezi kuřáky a nekuřáky byl statisticky významný rozdíl jen u diabetiků 2. typu, a to u parametru ztráta úponu;
7. osoby bez diabetu s obezitou a nadváhou (a to zejména ženy) měly významně vyšší hodnotu PI a obézní pak i vyšší hodnotu GI než jedinci s normální váhou. U diabetiků jsme rozdílné hodnoty PI prokázali pouze u žen s T2DM. U obézních diabetiček 2. typu jsme dále zjistili hlubší sondáž parodontálních chobotů. Nepodařilo se nám však prokázat, že obézní osoby trpí častěji pokročilou parodontitidou P3.

V mikrobiologické části studie jsme zjistili, že:

8. rozdíl v zastoupení bakteriálních druhů a v celkovém množství subgingivální mikroflóry u diabetiků oproti kontrolám byl minimální. U diabetiků 2. typu jsme však při porovnání s osobami bez diabetu zjistili významně vyšší četnost bakterií *P. micra* a *F. nucleatum*;
9. u diabetiků 2. typu jsme zjistili vyšší riziko resorpce alveolární kosti (test StomaGene®) než u kontrol.

LITERATURA

1. **Álamo, S. M., Soriano, Y. J., Sarrión Pérez, M. G.:** Dental considerations for the patient with diabetes. J. Clin. Exp. Dent., roč. 3, 2011, č. 1, s. 25–30.

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 1,
s. 3-12

2. **Apoorva, S. M., Sridhar, N., Suchetha, A.:** Prevalence and severity of periodontal disease in type 2 diabetes mellitus (non-insulin-dependent diabetes mellitus) patients in Bangalore city: an epidemiological study. J. Indian Soc. Periodontol., roč. 17, 2013, č. 1, s. 25–29.
3. **Borgnakke, W. S., Ylöstalo, P. V., Taylor, G. W., Genco, R. J.:** Effect of periodontal disease on diabetes: systematic review of epidemiologic observational evidence. J Clin Periodontol, roč. 40, 2013, suppl. 14, s. 135–152.
4. **Borilova Linhartova, P., Kastovsky, J., Lucanova, S., Bartova, J., Poskerova, H., Vokurka, J., Fassmann, A., Kankova, K., Izakovicova Holla, L.:** Interleukin-17A gene variability in patients with type 1 diabetes mellitus and chronic periodontitis: its correlation with IL-17 levels and the occurrence of periodontopathic bacteria. Mediators Inflamm., 2016, 2979846.
5. **Bořilová Linhartová, P., Valová, S., Kaštovský, J., Poskerová, H., Vokurka, J., Fassmann, A., Izakovičová Hollá, L.:** Genová variabilita v imunoregulačních faktorech u pacientů s chronickou parodontitidou a diabetes mellitus. Čes. Stomat., Prakt. zub. Lék., roč. 115–63, 2015, č. 4, s. 89–97.
6. **Campus, G., Salem, A., Uzzau, S.:** Diabetes and periodontal disease: a case-control study. J. Periodontol., roč. 76, 2005, č. 3, s. 418–425.
7. **Chaffee, B. W., Weston, S. J.:** Association between chronic periodontal disease and obesity: a systematic review and meta-analysis. J. Periodontol., roč. 81, 2010, č. 12, s. 1708–1724.
8. **Chapple, I. L. C., Genco, R.:** Diabetes and periodontal diseases: consensus report of the Joint EFP/AAP Workshop on periodontitis and systemic diseases. J. Clin. Periodontol., roč. 40, 2013, Suppl. 14, s. 106–112.
9. **Chávarry, N. G. M., Vettore, M. V., Sansone, C., Sheiham, A.:** The relationship between diabetes mellitus and destructive periodontal disease: a meta-analysis. Oral Health Prev. Dent., roč. 7, 2009, č. 2, s. 107–127.
10. **Dušková, J.:** Stomatologická onemocnění u diabetu. 1. vyd., Professional Publishing, Praha, Czechia, 2000, 56 s. ISBN: 80-86419-05-3.
11. **Dušková, J.:** Riziko diabetu pro onemocnění dutiny ústní. Med. Rev., roč. 7, 2001, č. 1, s. 12–14.
12. **Dušková, J., Broukal, Z.:** Vztah diabetes mellitus a onemocnění parodontu se zřetelem k mikrobiologickým nálezům v dutině ústní. (Literární přehled). Čs. Stomat., roč. 92, 1992, č. 6, s. 347–349.
13. **Dušková, J., Broukal, Z.:** Vztah onemocnění parodontu a diabetu – metaanalýza výsledků průřezových a intervenčních studií. Prakt. Lék., roč. 84, 2004, č. 10, s. 556–563.
14. **Dye, B. A., Genco, R. J.:** Tooth loss, pocket depth, and HbA1c information collected in a dental care setting may improve the identification of undiagnosed diabetes. J. Evid.-Based Dent. Pract., roč. 12, 2012, suppl. 3, s. 12–14.
15. **Ebersole, J. L., Hol, S. C., Hansard, R., Novak, M. J.:** Microbiologic and immunologic characteristics of periodontal disease in Hispanic Americans with type 2 diabetes. J. Periodontol., roč. 79, 2008, č. 4, s. 637–646.
16. **Engebretson, S., Kocher, T.:** Evidence that periodontal treatment improves diabetes outcomes: a systematic review and meta-analysis. J. Clin. Periodontol., roč. 40, 2013, suppl. 14, s. 153–163.
17. **Fagard, R. H., Nilsson, P. M.:** Smoking and diabetes – the double health hazard! Prim. Care Diabetes, roč. 3, 2009, č. 4, s. 205–209.
18. **Friedecký, B., Kratochvíla, J.:** Diabetes mellitus – laboratorní diagnostika a sledování stavu pacientů. http://www.cskb.cz/res/file/doporuceni/DM/DM_dop_201601.pdf. Přístup: 26. červenec 2016
19. **Garcia, D., Tarima, S., Okunseri, C.:** Periodontitis and glycemic control in diabetes: NHANES 2009 to 2012. J. Periodontol., roč. 86, 2015, č. 4, s. 499–506.
20. **Hintao, J., Teanpaisan, R., Chongsuvivatwong, V.:** The microbiological profiles of saliva, supragingival and subgingival plaque and dental caries in adults with and without type 2 diabetes mellitus. Oral Microbiol. Immunol., roč. 22, 2007, č. 3, s. 175–181.
21. **Holtfreter, B., Albandar, J. M., Dietrich, T.:** Standards for reporting chronic periodontitis prevalence and severity in epidemiologic studies: proposed standards from the Joint EU/USA Periodontal Epidemiology Working Group. J. Clin. Periodontol., roč. 42, 2015, č. 5, s. 407–412.
22. **Jagannathachary, S., Kamaraj, D.:** Obesity and periodontal disease. J. Indian Soc. Periodontol., roč. 14, 2010, č. 2, s. 96–100.
23. **Jansson, H., Lindholm, E., Lindh, C.:** Type 2 diabetes and risk for periodontal disease: a role for dental health awareness. J. Clin. Periodontol., roč. 33, 2006, č. 6, s. 408–414.
24. **Khader, Y. S., Albashaireh, Z. S. M., Hammad, M. M.:** Periodontal status of type 2 diabetics compared with nondiabetics in north Jordan. East Mediterr Health J Rev Santé Méditerranée Orient Al-Majallah Al-Sihhiyah Li-Sharq Al-Mutawassit, roč. 14, 2008, č. 3, s. 654–661.
25. **Khader, Y. S., Dauod, A. S., El-Qaderi, S. S.:** Periodontal status of diabetics compared with nondiabetics: a meta-analysis. J. Diabetes Complications, roč. 20, 2006, č. 1, s. 59–68.
26. **Lalla, E., Kaplan, S., Chang, S. J.:** Periodontal infection profiles in type 1 diabetes. J. Clin. Periodontol., roč. 33, 2006, č. 12, s. 855–862.
27. **Lim, L. P., Tay, F. B. K., Sum, C. F., Thai, A. C.:** Relationship between markers of metabolic control and inflammation on severity of periodontal disease in patients with diabetes mellitus. J Clin Periodontol, roč. 34, 2007, č. 2, s. 118–123.
28. **Löe, H.:** The gingival index, the plaque index and the retention index systems. J. Periodontol., roč. 38, 1967, suppl. 6, s. 610–616.
29. **Löe, H.:** Periodontal disease. The sixth complication of diabetes mellitus. Diabetes Care, roč. 16, 1993, č. 1, s. 329–334.
30. **Mathur, L. K., Manohar, B., Shankarapillai, R., Pandya, D.:** Obesity and periodontitis: a clinical study. J. Indian Soc. Periodontol., roč. 15, 2011, č. 3, s. 240–244.
31. **Moore, P. A., Weyant, R. J., Mongelluzzo, M. B.:** Type 1 diabetes mellitus and oral health: assessment of periodontal disease. J. Periodontol., roč. 70, 1999, č. 4, s. 409–417.
32. **Morita, I., Okamoto, Y., Yoshii, S.:** Five-year incidence of periodontal disease is related to body mass index. J. Dent. Res., roč. 90, 2011, č. 2, s. 199–202.
33. **Nishida, N., Tanaka, M., Hayashi, N.:** Determination of smoking and obesity as periodontitis risks using the classification and regression tree method. J. Periodontol., roč. 76, 2005, č. 6, s. 923–928.
34. **Rajhans, N. S., Kohad, R. M., Chaudhari, V. G., Mhaske, N. H.:** A clinical study of the relationship between diabetes mellitus and periodontal disease. J. Indian Soc. Periodontol., roč. 15, 2011, č.4, s. 388–392.
35. **Sarem, I. A., Nelson, R. G., Tulloch-Reid, M.:** Periodontal disease and mortality in type 2 diabetes. Diabetes Care, roč. 28, 2005, č. 1, s. 27-32.
36. **Shultis, W. A., Weil, E. J., Looker, H. C.:** Effect of periodontitis on overt nephropathy and end-stage renal disease in type 2 diabetes. Diabetes Care, roč. 30, 2007, č. 2, s. 306–311.

ČESKÁ STOMATOLOGIE
ročník 118,
2018, 1,
s. 3–12

37. **Soory, M.:** Hormone mediation of immune responses in the progression of diabetes, rheumatoid arthritis and periodontal diseases. Curr. Drug Targets Immune Endocr. Metab. Disord., roč. 2, 2002, č. 1, s. 13–25.
38. **Syrjälä, A. M. H., Ylöstalo, P., Niskanen, M. C., Knuuttila, M. L. E.:** Role of smoking and HbA1c level in periodontitis among insulin-dependent diabetic patients. J. Clin. Periodontol., roč. 30, 2003, č. 10, s. 871–875.
39. **Taylor, J. J., Preshaw, P. M., Lalla, E.:** A review of the evidence for pathogenic mechanisms that may link periodontitis and diabetes. J. Clin. Periodontol., roč. 40, 2013, suppl. 14, s. 113–134.
40. **Tsai, C., Hayes, C., Taylor, G. W.:** Glycemic control of type 2 diabetes and severe periodontal disease in the US adult population. Community Dent. Oral Epidemiol., roč. 30, 2002, č. 3, s. 182–192.
41. **Wiebe, C. B., Putnins, E. E.:** The periodontal disease classification system of the American Academy of Periodontology – an update. J. Can. Dent. Assoc., roč. 66, 2000, č. 11, s. 594–597.
42. **Yuan, K., Chang, C. J., Hsu, P. C.:** Detection of putative periodontal pathogens in non-insulin-dependent diabetes mellitus and non-diabetes mellitus by polymerase chain reaction. J. Periodontal. Res., roč. 36, 2001, č. 1, s. 18–24.

Článek vznikl s podporou grantu GAČR GB14-37368G, projektu Specifický výzkum MUNI/A/0948/2016 a z prostředků poskytnutých Lékařskou fakultou MU juniorskému výzkumníkovi Petře Bořilové Linhartové.

MUDr. Hana Poskerová, Ph.D.
Parodontologické oddělení
Stomatologická klinika
LF MU a FNUSA
Pekařská 53
656 91 Brno
e-mail: hana.poskerova@fnusa.cz

Attachment 12 Article "Oral diseases in diabetic patients"

ISSN 0042-773X (print) ISSN 1801-7592 (online) www.vnitrnilekarstvi.eu

časopis České internistické společnosti a Slovenskej internistickej spoločnosti

62 let ve službách interní medicíny

Brno, 6. prosince 2018

Vážená paní doktorko,

dovoluji si Vám sdělit, že práce autorského kolektivu Poskerová, Bořilová Linhartová, Izakovičová Hollá: **„Onemocnění dutiny ústní u pacientů s diabetem"** byla přijata k publikaci v časopise Vnitřní lékařství, v monotematickém čísle časopisu **„Diabetologie I."**.

V současné době Vám ale nejsem schopen jednoznačně říci, do kterého čísla ročníku 2019 časopisu Vnitřní lékařství bude výše uvedená práce zařazena.

O přesném zařazení výše uvedené práce do konkrétního čísla časopisu Vnitřní lékařství Vás budu ještě samozřejmě elektronicky informovat.

Se srdečnými pozdravy a s přáním klidného adventního času

Prim. MUDr. **Petr Svačina**, v.r.,
šéfredaktor časopisu Vnitřní lékařství

Vážená paní
MUDr. Hana P o s k e r o v á, Ph.D.,

Stomatologická klinika LF MU a FN u sv. Anny,
Pekařská 53,
656 91 B r n o.
e-mail: hana.pskerova@fnusa.cz

POZOR! Od 1. ledna 2016 platí na vedoucího odborného redaktora jen telefonní číslo 603420260, dále neplatí maily psvacina@vez.brn.justice.cz a petr.svacina@fnusa.cz, ale nově kromě mailového spojení svacinavl@seznam.cz také nový mail svacinavl@hotmail.com.

vedoucí odborný redaktor
prim. MUDr. Petr Svačina
II. interní klinika LF MU a FN u sv. Anny
Pekařská 53 | 656 91 Brno
E | svacinavl@seznam.cz,
svacinavl@hotmail.com
M | +420 603 420 260

odpovědný redaktor
dr. Boris Skalka
E | boris.skalka@fa-ma.cz
M | +420 737 985 593

nakladatel

Srbská 2186/19 | 612 00 Brno

Redakce
Facta Medica, s.r.o.
Jana Babáka 2733/11 | 612 00 Brno
E | fama-redakce@fa-ma.cz
M | +420 737 287 512

vydavatel

Onemocnění dutiny ústní u pacientů s diabetem

(Přehledný referát)

Oral diseases in diabetic patients

(Review of Literature)

Poskerová H.[1], Bořilová Linhartová P.[1, 2], Izakovičová Hollá L.[1, 2]

[1] Stomatologická klinika LF MU a FN u sv. Anny, Brno

[2] Ústav patologické fyziologie LF MU a FN, Brno

[1]Clinic of Stomatology, FM MU and St. Anne's Faculty Hospital, Brno

[2]Department of Pathophysiology, FM MU and Faculty Hospital Brno

Souhrn:

Autoři ve svém sdělení shrnují současné poznatky o vzájemných souvislostech mezi onemocněním diabetes mellitus (DM) a chorobami tkání dutiny ústní. Zabývají se vlivem diabetu na stav tvrdých zubních tkání, ústních sliznic a slinných žláz a vysvětlují, jakým způsobem k těmto nežádoucím změnám dochází. Zaměřují se především na oboustranný vztah mezi diabetem a parodontitidou, na společné patogenetické znaky obou chorob a na vliv parodontologické léčby na základní metabolické onemocnění. Dokládají, že diabetes na jedné straně negativně ovlivňuje stav parodontálních tkání, na straně druhé je však i zánětlivé onemocnění parodontu rizikovým faktorem pro vznik hyperglykemie. Autoři poukazují na vznikající bližší spolupráci mezi diabetology a parodontology (Perio-Diabetes Workshop v roce 2017) a zamýšlejí se nad možnostmi účinné prevence a lepší motivace diabetiků k péči o orální zdraví.

Klíčová slova: diabetes mellitus, gingivitis, parodontitis, ústní dutina, zánětlivé mediátory

Summary:

In this paper, the authors summarize current findings on the relationship between diabetes mellitus (DM) and oral tissue diseases. They study the effect of diabetes on the condition of hard dental tissues, oral mucosa and salivary glands and explain how these undesirable changes occur. They focus primarily on the bilateral relationship between diabetes and periodontitis, common pathogenetic traits of both diseases and on the effect of periodontal

treatment on basic metabolic diseases. They show that diabetes not only negatively affects the condition of periodontal tissues, but inflammatory disease of periodontium is also a risk factor for hyperglycemia. The authors point to the emerging closer co-operation between diabetologists and parodontologists (Perio-Diabetes Workshop in 2017) and consider possibilities of effective prevention and better motivation of diabetics for oral health care.

Key words: diabetes mellitus, gingivitis, periodontitis, oral cavity, inflammatory mediators

Úvod

Diabetes mellitus je závažné metabolické onemocnění provázené řadou komplikací, které často výrazně snižují kvalitu života diabetiků. Kromě klasických komplikací má diabetes vliv také na tkáně dutiny ústní. Orální projevy DM se týkají zejména parodontu, ale i sliznic dutiny ústní, slinných žláz a tvrdých zubních tkání. V posledních letech se výzkum v této oblasti zaměřuje především na objasnění souvislostí mezi DM a parodontitidou, přičemž se ukazuje, že vztah mezi oběma chorobami je oboustranný. V letech 2012 a 2017 proběhla ve Španělsku dvě významná pracovní setkání evropských a amerických odborníků, kteří systematicky vyhodnotili stovky studií z medicínských databází a následně zveřejnili výsledky o současném pohledu na souvislost mezi oběma chorobami. Tyto aktivity by měly vést k lepší spolupráci mezi všeobecnými lékaři a stomatology/zubními lékaři a zvýšit informovanost o dané problematice u všech zainteresovaných skupin obyvatelstva.

Projevy diabetu v dutině ústní (tvrdé zubní tkáně, ústní sliznice, slinné žlázy)

Změny v dutině ústní většinou, podobně jako i ostatní chronické komplikace diabetu, korespondují s délkou trvání onemocnění, úrovní jeho kompenzace a přítomností dalších systémových změn.

O vlivu diabetu na vyšší kazivost chrupu se vedou četné diskuze, řada výzkumných prací však vyšší výskyt **zubního kazu** u diabetiků potvrzuje [1,2]. Předpokládanou příčinou je nižší salivace a snížená pufrovací kapacita sliny (důsledkem postižení slinných žláz), zvýšená hladina glukózy ve slině a v sulkulární tekutině (vlivem hyperglykemie), ale také vyšší frekvence příjmu potravy [3].

Nedostatečná tvorba slin a z ní vyplývající xerostomie (subjektivní pocit suchosti v ústech), postihuje řadu diabetiků[4,5]. Na vznik hyposialie má zřejmě vliv polyurie, poruchy mikrocirkulace, narušení bazálních membrán žlázového epitelu, ale i vedlejší účinky

některých léčiv[3]. Snížené množství slin vede ke zvýšené akumulaci zubního plaku, k atrofii ústní sliznice (zejména **atrofické glossitidě**) a snazší tvorbě dekubitů pod snímacími zubními náhradami. Hyposialie souvisí společně se sníženou imunitou a vyšší hladinou glukózy ve slinách s projevy **orální kandidózy** (obr. 1). U diabetiků (zejména nedostatečně kompenzovaných) se vyskytuje častěji, než u zdravé populace [6,7] a je lokalizovaná většinou v ústních koutcích (**stomatitis angularis**), na hřbetu jazyka nebo na tvrdém patře u pacientů se snímací zubní náhradou. Další komplikace, které diabetiky v dutině ústní postihují, jsou rychlé šíření **odontogenní infekce**[7], porucha hojení měkkých tkání a extrakčních ran[7,8]. Neobjasněné pálení ústních sliznic, případně poruchy chuti u diabetiků popisujeme jako **stomatodynie a glosodynie** a při jejich vzniku předpokládáme vliv periferní diabetické neuropatie[7,8]. Tyto obtíže mohou přispívat k narušení stravovacích návyků, ke zvýšenému příjmu jídla a následně k obezitě. U diabetiků také častěji diagnostikujeme nezánětlivou hypertrofii žlázového parenchymu příušní slinné žlázy - **sialoadenózu** ve formě oboustranného asymptomatického zduření příušních slinných žláz (Charvátův příznak). Kombinace diabetu s hypertenzí a postižením sliznic dutiny ústní ve formě orálního lichenu je označovaná jako **Grinspanův syndrom** (obr. 2). U diabetiků 2. typu (DM2) lichenoidní změny ústní sliznice mohou vznikat jako poléková rekce (antihypertenziva a perorální antidiabetika)[9]. Někteří autoři považují diabetes také za rizikový faktor pro vznik **premaligních změn a tumorů** v dutině ústní[10].

Projevy diabetu v oblasti parodontu

Diabetes přispívá zejména ke vzniku parodontitidy, kterou popsal Löe v roce 1993 ve své rozsáhlé studii jako „šestou komplikaci diabetu“[11] . Dušková a Broukal uvádějí v jednom ze svých sdělení, že souvislost mezi oběma chorobami byla známa již koncem devatenáctého století, přičemž destruktivní onemocnění parodontu bylo často zjištěno zubním lékařem dříve, než byla diagnostikována základní metabolická porucha[12].

Intenzivní výzkum sledující výskyt gingivitidy (zánět dásní) a parodontitidy (zánět závěsného aparátu zubu) u diabetiků probíhá zejména od šedesátých let dvacátého století. Zaměřuje se především na diabetes 2. typu, protože diabetici 1. typu bývají diagnostikováni a vyšetřováni hlavně v dětství (zejména ve skupině 11-15 let), kdy se parodontitida ještě nemusí projevit. V tomto věku je popisována vyšší prevalence gingivitid, a to až dvakrát častější než u zdravých dětí a adolescentů a jejich těžší forma[13]. Juvenilní diabetik zřejmě reaguje

zánětem dásní již na takové množství mikrobiálního zubního povlaku, které by u celkově zdravého jedince gingivitidu ještě nevyvolalo[14].
Z výzkumů dospělých diabetiků jasně vyplývá, že DM zvyšuje incidenci, prevalenci, progresi a závažnost parodontitidy a stává se tak významným rizikovým faktorem pro onemocnění parodontálních tkání. Potvrzuje to nejenom rozsáhlá, dnes již klasická práce Löe[11] (vyšetřil 2180 osob; pokročilou parodontitidou ve věku 15-35 let trpělo 45 % mužů a 48 % žen s DM2 ve srovnání s 8 % osob bez diabetu), ale i nejnovější rozsáhlá meta-analytická studie[15] .

K poškození parodontálních tkání přispívá především dlouhodobá hyperglykemie a její následky. Pozdní produkty glykace proteinů (AGEs) spolu s dalšími patogenetickými mechanizmy (např. oxidační stres, akumulace polyolů), vedou ke strukturálním a funkčním změnám kapilár gingiválního plexu, k poruchám metabolizmu kolagenu gingivální tkáně a parodontálních ligament, ke změnám funkce imunitních buněk a k poruchám metabolizmu alveolární kosti[16–19]. Takto oslabený parodont je potom náchylnější k působení mikroorganizmů dentálního plaku a umožňuje rozvinutí jejich patogenního potenciálu[16,19] (schéma 1).
Závažným stavem při léčbě diabetu je kromě hyperglykemie také hypoglykemie. Japonští autoři sledovali vliv iatrogenně navozené hypoglykemie na parodontální tkáně, významný vliv prokázali zejména na buňky parodontálních ligament[20]. Pokud je kultivovali v prostředí s nadbytkem glukózy, byla narušena jejich schopnost adheze a pohybu. V hypoglykemickém prostředí však buňky parodontálních ligament postupně podlehly buněčné smrti. Z výsledků této studie vyplývá, že časté výkyvy glykemie mohou buňky parodontu vážně poškodit.

Postižení parodontu tedy souvisí zejména s tím, jak je diabetik kompenzovaný. S narůstající délkou trvání diabetu a s nástupem pozdních cévních komplikací zesiluje vliv metabolické choroby také na průběh a závažnost parodontitidy. Nejméně patrný je vliv diabetu na parodont u dobře kompenzovaných diabetiků 2. typu, u kterých rozsah zánětlivého postižení závěsného aparátu zubů závisí především na úrovni jejich ústní hygieny[21]. Velká část paceintů s DM2 se navíc léčí s vysokým krevním tlakem. Pokud jsou v medikaci hypertenze použity blokátory kalciových kanálů (zejména nifedipin), může se u pacientů s nedostatečnou ústní hygienou rozvinout plakem podmíněná léky modulovaná hyperplastická gingivitida (obr. 3ab,4).

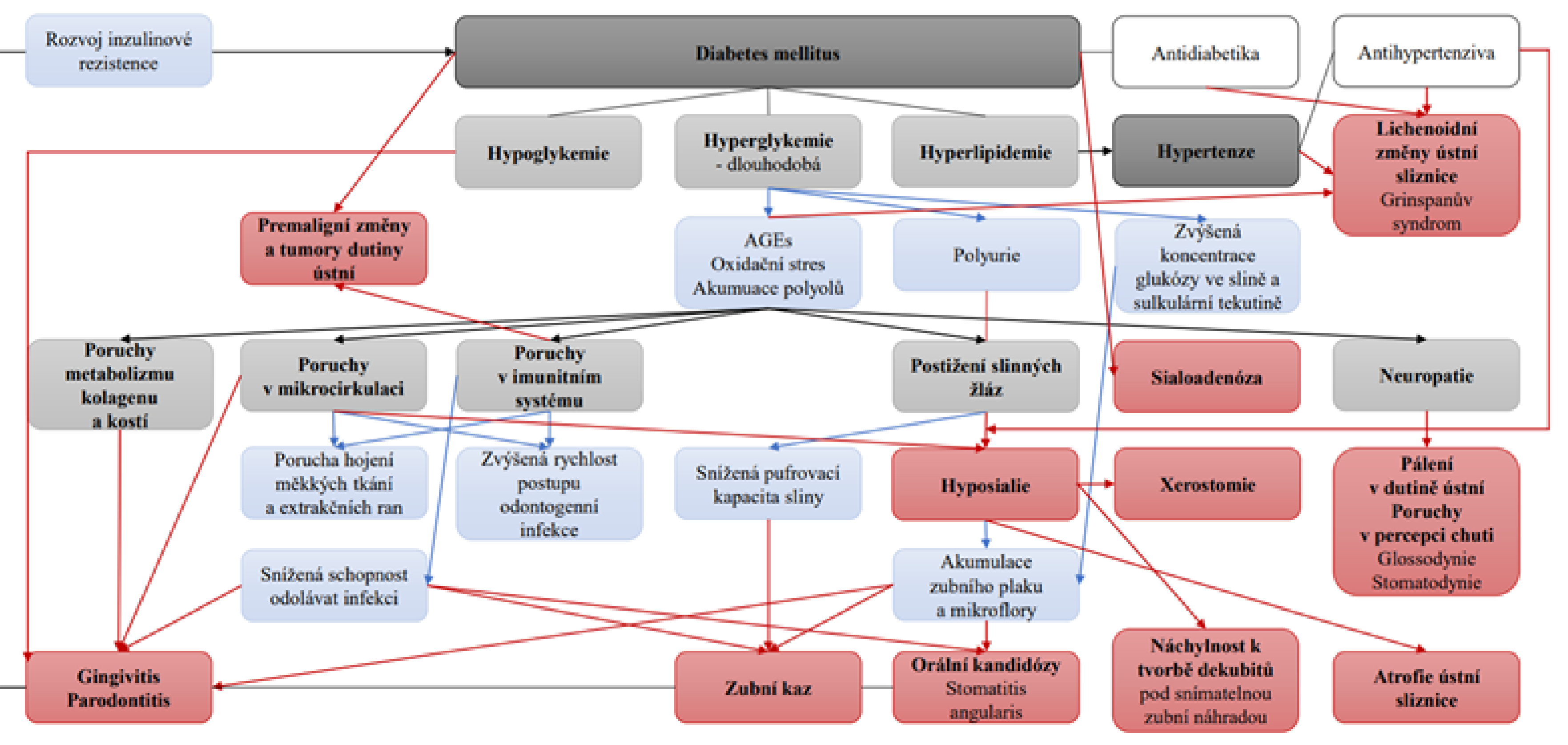


Schéma 1.: Vzájemná souvislost mezi diabetem a parodontitidou [1,2,].

Vliv chronických zánětlivých parodontopatií na vznik a rozvoj diabetu

Parodontitida je chronické zánětlivé onemocnění závěsného aparátu zubů. Tento zánět vzniká a je dále udržován dlouhodobou přítomností zubního mikrobiálního povlaku v oblasti marginální gingivy (nad i pod okrajem dásně). Způsobuje destrukci celého závěsného aparátu zubů včetně úbytku alveolární kosti a tvorby pravých parodontálních chobotů.
Parodontální bakterie (zejména gram-negativní anaerobní mikroorganizmy) a jejich virulentní faktory mohou pronikat do krevního oběhu, což vede k systémové odezvě[19]. Trvalé uvolňování bakteriálních lipopolysacharidů, enzymů a exotoxinů v oblasti gingivy indukuje imunitní odpověď, čímž se zvyšuje exprese prozánětlivých mediátorů (zejména interleukinů - IL-1β, IL-6, tumor nekrotizujícího faktoru-TNF-α a prostaglandinů-PGE) řada z nich se dostává do krevního oběhu a dále tak přispívá k udržování systémového zánětu[19,22,23]. Mediátory zánětu pak spouštějí kaskádu složitých dějů, které ovlivňují glukózový a lipidový metabolizmus, indukují inzulinovou rezistenci[16,19,22,23] a mohou způsobit i poškození beta buněk pankreatu[24] (schéma 1).

Demmer a jeho spolupracovníci analyzovali v rámci národní studie NHANES (National Health and Nutrition Examination Survey) rozsáhlý vzorek populace (9296 jedinců)[25]. Zjistili, že ve skupině se středně pokročilou parodontitidou mělo diabetes 2,26 krát více osob, než ve skupině parodontologicky zdravých jedinců. Stejní autoři v prospektivní pětileté studii sledovali vliv neléčené parodontitidy na změny hodnot glykovaného hemoglobinu u 2973 osob bez diabetu[26]. Prokázali, že u těch, kteří trpěli na počátku těžkou pokročilou parodontitidou, stoupla hladina tohoto ukazatele během sledovaného období pětkrát ve srovnání s osobami bez parodontitidy. Toto byla první práce, která doložila, že zánětlivé onemocnění parodontu může vést ke zvýšení glykemie i u osob bez diabetu.

Z řady studií za posledních dvacet let vyplývá, že diabetici s těžkou formou parodontitidy jsou náchylnější ke vzniku a rozvoji diabetických komplikací [23,27] a je zřejmé, že prevalence a závažnost těchto onemocnění koreluje se stupněm parodontitidy[11,28]. Saremi popsal 8,5 krát vyšší úmrtnost na diabetickou nefropatii a 2,3 krát vyšší úmrtnost na ischemickou chorobu srdeční u diabetiků s těžkou parodontitidou než u těch se zdravým parodontem případně mírnou formu zánětu[28].

Vliv parodontologické léčby na glykemický profil

Parodontologická léčba spočívá v pravidelné a trvalé kontrole mikrobiálního zubního plaku. Zahrnuje odstranění viditelného zubního kamene nad dásní a v léčbě parodontálních chobotů ve formě subgingiválního ošetření (odstranění zubního kamene a plaku pod okrajem dásně a vyhlazení povrchové vrstvičky nekrotického zubního cementu). Nezbytně nutná je spolupráce pacienta při každodenní ústní hygieně, aby nedošlo k rychlé rekolonizaci očištěných míst patogenními mikroorganizmy.

Řada studií potvrdila, že efektivní parodontologická léčba doprovázená pravidelným a dostatečným odstraňováním zubního plaku v rámci domácí péče může snížit hladinu glykovaného hemoglobinu, a to zejména u nedostatečně kompenzovaných diabetiků s pokročilou parodontitidou[17,29–31]. Ke zlepšení glykemického profilu pacienta vede zřejmě pokles systémového zánětu a snížení hladin zánětlivých mediátorů v návaznosti na redukci zánětu parodontu po léčbě[32].

Obezita a parodontitida – vzájemné ovlivňování

V posledních desetiletích se sleduje i vztah mezi obezitou a parodontitidou. V patogeneze toho procesu hraje roli řada mechanizmů, zejména vliv obezitou indukovaných zánětlivých mediátorů, které jsou produkovány viscerální tukovou tkání. Následná infiltrace tkání makrofágy a dalšími buňkami, které uvolňují prozánětlivé mediátory, vede k udržování recipročního cyklu chronického zánětu. Lipopolysacharidy parodontálních patogenů podporují např. sekreci TNF-α tukovou tkání, což na jedné straně způsobuje poškození parodontálních tkání a na druhé straně přispívá ke vzniku inzulinové rezistence a vede k dysfunkci beta buněk pankreatu[24,33,34]. Obezita je tak významným rizikovým faktorem pro vznik parodontitidy i bez přítomného diabetu a naopak zánětlivé onemocnění parodontu může zhoršit metabolický syndrom, jehož je obezita součástí (schéma 1). Levine popisuje tuto situaci jako trojúhelníkový vztah obezita – diabetes – parodontitida, přičemž dochází k vzájemnému ovlivnění těchto chorob[35].

Ve snaze o objasnění vlivu parodontitidy na celkový zdravotní stav se v roce 2012 ve Španělsku konala pracovní konference (9th European Workshop in Periodontology), na které se přední evropští a američtí parodontologové zaměřili na studium vztahů mezi parodontitidou a systémovými chorobami. Odborníci pracovali ve čtyřech skupinách cílených na DM, kardiovaskulární choroby, těhotenství a některá další onemocnění a systematicky vyhodnotili

stovky původních výzkumných prací zveřejněných v medicínských databázích. V dubnu roku 2013 vydaly obě odborné společnosti sborník konference, ve kterém byly shrnuty závěry jejich systematického zkoumání. Celkem čtyři souhrnné práce se týkají diabetu[19,23,27,36]. Poskytují komplexní souhrn dosavadních znalostí o vztazích mezi diabetem a parodontitidou a vyvozují tyto tři základní teze:

1. u osob s parodontitidou pronikají parodontální bakterie a jejich virulentní faktory do krevního oběhu, což vede k systémovému zánětu organizmu. Tento mechanizmus může mít vliv na rozvoj diabetu a jeho komplikací[19].
2. pokročilá parodontitida ovlivňuje hladinu glykemie u diabetiků i u osob bez diabetu. Existuje přímá souvislost mezi závažností parodontitidy a výskytem diabetických komplikací a také zvýšené riziko vzniku diabetu u pacientů s pokročilou parodontitidou[27].
3. z randomizovaných klinických studií vyplynulo, že konzervativní parodontologické ošetření může vést k redukci hladiny glykovaného hemoglobinu (HbA_{1c}) v průměru o 0,36 % při kontrole za 3 měsíce, což je klinicky významný výsledek[36].

Na tuto konferenci po pěti letech navázalo pracovní setkání Perio-Diabetes Workshop konané v roce 2017 opět ve Španělsku. Účastníky byli tentokrát kromě parodontologů také diabetologové z International Diabetes Federation. Jejich cílem bylo aktualizovat poznatky z předchozí konference na základě analýzy nových výzkumných studií z let 2013 – 2017. Z meta-analytického šetření vyplynula následující zjištění[22,37–39]:

1. osoby bez diabetu s parodontitidou měly vyšší hladinu HbA_{1c} v séru v porovnání s těmi se zdravým parodontem[37].
2. osoby s parodontitidou měly větší pravděpodobnost rozvoje prediabetu nebo DM2 [37].
3. většina studií se shodla na tom, že parodontitida je rizikový faktor pro rozvoj diabetických komplikací[37].
4. u diabetiků s parodontitidou byla zjištěna horší funkce beta buněk pankreatu, větší oxidační stres, závažnější dyslipidemie a vyšší hladina cirkulujících prozánětlivých mediátorů (zejména TNF-α a C-reaktivního proteinu) než u osob s diabetem a zdravým parodontem[22].

5. parodontologická léčba spolu s dostatečnou domácí péčí vedla ke zlepšení stavu parodontu i u diabetiků s nedostatečně kompenzovaným diabetem[38].
6. úspěšná parodontologická léčba u diabetiků redukovala hladiny TNF-α a CRP[22]
7. konzervativní terapie parodontu vedla ke statisticky signifikantnímu snížení hladiny HbA_{1c} v séru (měřeno za 3-4 měsíce po léčbě), a to i u nedostatečně kompenzovaných diabetiků 2. typu. Rozsah redukce HbA_{1c} se v této meta-analytické studii pohyboval v rozmezí 0,27 % - 0,48 %[38] . Pro diabetiky 1. typu nebyly k dispozici dostatečně relevantní studie.

Podobně jako na předchozí konferenci v roce 2012 také autoři loňského pracovního setkání Perio-Diabetes Workshop závěrem navrhli některá opatření, která by mohla zlepšit informovanost a posléze i zdravotní stav pacientů[39]:

A/ Doporučení pro všeobecné lékaře a další profesionály v péči o diabetiky:

- vzdělávací programy pro diabetiky by měly zahrnovat i edukaci týkající se orálního zdraví.
- diabetici by měli být informováni o tom, že parodontitida má negativní vliv na kompenzaci diabetu a může zvýšit riziko diabetických komplikací.
- diabetolog by se měl svých pacientů tázat na jejich orální zdraví a ptát se i na případné symptomy parodontitidy (krvácení/hnisavá exsudace z dásní, posuny zubů, ztráta zubů, halitóza).
- všichni nově diagnostikovaní diabetici by měli být v rámci komplexního přístupu odesláni k prohlídce u zubního lékaře (která by měla zahrnovat i základní vyšetření parodontu).
- diabetici by měli být informováni o dalších možných problémech v dutině ústní (suchost a pálení sliznic, zvýšené riziko kvasinkové infekce, zhoršené hojení ran).

B/ Doporučení pro diabetické pacienty v ambulancích všeobecných lékařů:

- informace pro pacienty o vztahu diabetes – parodontitis.
- doporučení pravidelně navštěvovat zubního lékaře.
- popis zaníceného parodontu (zarudnutí/otok/ krvácení z dásní, zubní kámen, posuny zubů, ztráta zubů), tyto příznaky však nemusí být vždy patrné (zejména krvácení z dásní nebývá manifestní u kuřáků).
- informace o tom, jak se naučit správným způsobem udržovat dobrou hygienu dutiny ústní včetně čištění mezizubních prostor.
- popis dalších možných problémů v dutině ústí (suchost a pálení sliznic, zvýšené riziko kvasinkové infekce, zhoršené hojení ran).

C/ Doporučení pro zubní lékaře, kteří pečují o diabetiky a o osoby s rizikovými faktory pro vznik diabetu:

- zubní lékař by měl diabetiky informovat o tom, že diabetes zvyšuje riziko vzniku a rozvoje parodontitidy a ovlivňuje zdraví dutiny ústní, na druhé straně parodontitida zvyšuje nebezpečí nedostatečné kompenzace diabetu a diabetických komplikací.
- zubní lékař by měl zjistit v rámci anamnézy typ diabetu, délku jeho trvání, kompenzaci a druh léčby včetně další medikace.
- u každého diabetika by měl být vyšetřen stav parodontu a diabetici se zánětlivými parodontopatiemi by měli být adekvátně parodontologicky léčeni.
- u diabetiků je nutné věnovat pozornost i dalším případným orálním komplikacím (hyposalivace s xerostomií, orální kandidóza, stomatodynie a glosodynie, zubní kaz).
- důležitá je motivace pacienta k řádné ústní hygieně (zubní mikrobiální povlak má vliv nejen na jejich orální, ale i na celkové zdraví) a individuální instruktáž dentálními pomůckami včetně mezizubních.
- diabetici, kteří již přišli o větší část chrupu, by měli být včas proteticky ošetřeni tak, aby nebyl negativně ovlivněn příjem potravy.
- osoby s rizikovými faktory pro vznik diabetu 2. typu (obezita, vysoký krevní tlak, zvýšená hladina cholesterolu, rodinná zátěž) by měli být zubním lékařem informováni o tomto riziku a měli by být odesláni k vyšetření praktickým lékařem.
- komplikovanější případy by měl zubní lékař konzultovat s diabetologem, například chirurgická léčba a aplikace zubních implantátů není vhodná u nedostatečně kompenzovaných diabetiků.

D/ Doporučení pro diabetické pacienty a osoby s rizikovými faktory pro diabetes v zubních ambulancích.

- diabetici by se u zubního lékaře/parodontologa měli dozvědět o vzájemném vztahu diabetes-parodontitis, i o možném vlivu jejich onemocnění na další části dutiny ústní
- důležité jsou zejména informace o domácí péči ve smyslu pravidelného a pečlivého odstraňování zubního plaku včetně mezizubního a o potřebnosti pravidelných stomatologických kontrol (i v případě, že pacient netrpí žádnými aktuálními obtížemi).

Význam dostatečné komunikace s pacienty a jejich informovanosti dokládají ve své práci thajští autoři, kteří zjišťovali vliv motivace a edukace na stav chrupu a kompenzaci diabetu [40]. Polovina studovaného souboru diabetiků získala rozsáhlé informace týkající se zdravého

životního stylu a provádění kvalitní ústní hygieny včetně individuálních konzultací a tištěného manuálu. Kontrolní skupina byla poučena o důležitosti těchto faktorů ovlivňujících zdraví pouze rutinním způsobem. Pacienti byli vyšetřeni na začátku studie, po třech a po šesti měsících. Ukázalo se, že současná změna životního stylu a ústní hygieny u dobře informovaných osob první skupiny vedla k lepším výsledkům týkajícím se kompenzace diabetu i stavu parodontu v porovnání s kontrolní skupinou.

Nizozemské autory zajímalo, jak vypadá spolupráce mezi stomatology a diabetology v péči o pacienty s diabetem[41]. Vybrali 889 pacientů navštěvujících diabetologické oddělení nemocnice v Amsterdamu, diabetolog měl za úkol předat svým pacientům dotazník týkající se stavu chrupu a parodontu pro jejich stomatologa a získat ho od nich při další návštěvě zpět. Dotazník zahrnoval výsledky běžného stomatologického vyšetření. Z celkového počtu 889 diabetiků pouze 408 osob dotazník od diabetologa skutečně obdrželo. Z těchto 408 dotazníků předaných stomatologovi se pouze 168 vrátilo vyplněných zpět, po telefonickém kontaktu se podařilo získat dalších 67. Autoři konstatují, že mnohé v komunikaci diabetolog – pacient – zubní lékař by bylo možné zlepšit. Na závěr poznamenávají, že na základě výsledků tohoto projektu se diabetologové v jejich nemocnici začali dotazovat svých pacientů na stav chrupu a dutiny ústní a doporučují jim pravidelné návštěvy stomatologa/zubního lékaře.

Závěr

Komplexní péče o diabetika vyžaduje interdisciplinární spolupráci. Výzkumy posledních let poukazují na významné souvislosti mezi diabetem a parodontitidou, mechanizmus jejich vzájemného působení je však dosud znám jen částečně. Přesto je možné relativně jednoduchými opatřeními, jakými jsou dobrá informovanost pacienta, důraz na pravidelnou stomatologickou péči a správnou hygienu dutiny ústní, dosáhnout mnohých zlepšení. Informace o efektu parodontologické léčby na pokles mediátorů zánětu a glykovaného hemoglobinu v řadě studií znamenají posun v pohledu na prevenci diabetu i jeho komplikací. Ukazuje se, že důsledná parodontologická léčba diabetiků by mohla přispět ke stabilizaci jejich celkového onemocnění. V posledních letech se také objevují doporučení, aby zubní lékaři věnovali zvýšenou pozornost rizikovým pacientům pro diabetes a odesílali je k dalšímu vyšetření praktickým lékařem. Z výše uvedeného vyplývá, že v komplexní péči o diabetiky je řada společných témat mezi všeobecnými a zubními lékaři a účinnější spolupráce mezi těmito profesemi by mohla vést ke zlepšení kvality života diabetických pacientů.

Článek vznikl s podporou grantu GAČR GB14-37368G, projektu Specifický výzkum MUNI/A/1008/2017 a z prostředků poskytnutých Lékařskou fakultou MU juniorskému výzkumníkovi Petře Bořilové Linhartové.

Literatura

1. Almusawi MA, Gosadi I, Abidia R, Almasawi M, Khan HA. Potential risk factors for dental caries in Type 2 diabetic patients. Int J Dent Hyg 2018; 11. doi: 10.1111/idh.12346.

2. Coelho A, Paula A, Mota M. Dental caries and bacterial load in saliva and dental biofilm of type 1 diabetics on continuous subcutaneous insulin infusion. J Appl Oral Sci 2018; 26. doi: 10.1590/1678-7757-2017-0500.

3. Álamo SM, Soriano YJ, Sarrión Pérez MG. Dental considerations for the patient with diabetes. J Clin Exp Dent 2011; 3(1): 25–30.

4. Molania T, Alimohammadi M, Akha O, Mousavi J, Razvini R, Salehi M. The effect of xerostomia and hyposalivation on the quality of life of patients with type II diabetes mellitus. Electron Physician 2017; 9(11): 5814–19.

5. Carramolino-Cuéllar E, Lauritano D, Silvestre F-J, Carinci F, Lucchese A, Silvestre-Rangil J. Salivary flow and xerostomia in patients with type 2 diabetes. J Oral Pathol Med 2018; 47(5): 526–30.

6. Mohammadi F, Javaheri MR, Nekoeian S, Dehghan P. Identification of Candida species in the oral cavity of diabetic patients. Curr Med Mycol 2016; 2(2): 1–7.

7. Al-Maskari AY, Al-Maskari MY, Al-Sudairy S. Oral Manifestations and Complications of Diabetes Mellitus: a review. Sultan Qaboos Univ Med J 2011; 11(2): 179–86.

8. Negrato CA, Tarzia O. Buccal alterations in diabetes mellitus. Diabetol Metab Syndr 2010; 2(2): 3. doi:10.1186/1758-5996-2-3.

9. Goyal L, Gupta ND, Gupta N. Grinspan syndrome with periodontitis: Coincidence or correlation? J Indian Soc Periodontol 2018; 22(3): 263–65.

10. Gong Y, Wei B, Yu L, Pan W. Type 2 diabetes mellitus and risk of oral cancer and precancerous lesions: a meta-analysis of observational studies. Oral Oncol 2015; 51(4): 332–40.

11. Löe H. Periodontal disease. The sixth complication of diabetes mellitus. Diabetes Care 1993; 16(1): 329–34.

12. Dušková J, Broukal Z. Vztah diabetes mellitus a onemocnění parodontu se zřetelem k mikrobiologickým nálezům v dutině ústní. (Literární přehled). Českoslov stomatol 1992; 92(6): 347–49.

13. Lalla E, Cheng B, Lal S, et al. Periodontal changes in children and adolescents with diabetes: a case-control study. Diabetes Care 2006; 29(2): 295–99.

14. Dušková J. Riziko diabetu pro onemocnění dutiny ústní. Medica rev 2001; 7(1): 12–14.

15. Nascimento GG, Leite FRM, Vestergaard P, Scheutz F, López R. Does diabetes increase the risk of periodontitis? A systematic review and meta-regression analysis of longitudinal prospective studies. Acta Diabetol 2018; 55(7): 653–67.

16. Rajhans NS, Kohad RM, Chaudhari VG, Mhaske NH. A clinical study of the relationship between diabetes mellitus and periodontal disease. J Indian Soc Periodontol 2011; 15(4): 388–92.

17. Balasundaram A, Ponnayian D, Parthasarathy H. Diabetes mellitus – a periodontal perspective. SRM University Journal of Dental Sciences 2010; 1(1): 79–85.

18. Gurav A, Jadhav V. Periodontitis and risk of diabetes mellitus. J Diabetes 2011; 3(1): 21–28.

19. Taylor JJ, Preshaw PM, Lalla E. A review of the evidence for pathogenic mechanisms that may link periodontitis and diabetes. J Clin Periodontol 2013; 40(Suppl 14): 113–34.

20. Nishimura F, Takahashi K, Kurihara M, Takashiba S, Murayama Y. Periodontal disease as a complication of diabetes mellitus. Ann Periodontol 1998; 3(1): 20–29.

21. Poskerová H, Bořilová Linhartová P, Vokurka J, Fassmann A, Izakovičová Hollá L. Diabetes mellitus a orální zdraví. Česká stomatologie a Praktické zubní lékařství : časopis stomatologických společností : časopis pro další vzdělávání pracovníků v péči o orální zdraví 2014; 114–62(5): 75–86.

22. Polak D, Shapira L. An update on the evidence for pathogenic mechanisms that may link periodontitis and diabetes. J Clin Periodontol 2018; 45: 150–66.

23. Chapple ILC, Genco R. Diabetes and periodontal diseases: consensus report of the Joint EFP/AAP Workshop on periodontitis and systemic diseases. J Clin Periodontol 2013; 40(Suppl 14): 106–12.

24. Ballak DB, Stienstra R, Tack CJ, Dinarello CA, van Diepen JA. IL-1 family members in the pathogenesis and treatment of metabolic disease: Focus on adipose tissue inflammation and insulin resistance. Cytokine 2015; 75(2): 280–90.

25. Demmer RT, Jacobs DR Jr, Desvarieux M. Periodontal disease and incident type 2 diabetes: results from the First National Health and Nutrition Examination Survey and its epidemiologic follow-up study. Diabetes Care 2008; 31: 1373–79.

26. Demmer RT, Desvarieux M, Holtfreter B, et al. Periodontal status and A1C change: longitudinal results from the study of health in Pomerania (SHIP). Diabetes Care 2010; 33(5): 1037–43.

27. Borgnakke WS, Ylöstalo PV, Taylor GW, Genco RJ. Effect of periodontal disease on diabetes: systematic review of epidemiologic observational evidence. J Clin Periodontol 2013; 40(Suppl 14): 135–52.

28. Saremi A, Nelson RG, Tulloch-Reid M, et al. Periodontal disease and mortality in type 2 diabetes. Diabetes Care 2005; 28(1): 27–32.

29. Liew AKC, Punnanithinont N, Lee Y-C, Yang J. Effect of non-surgical periodontal treatment on HbA1c: a meta-analysis of randomized controlled trials. Aust Dent J 2013; 58(3): 350–57.

30. Sgolastra F, Severino M, Pietropaoli D, Gatto R, Monaco A. Effectiveness of periodontal treatment to improve metabolic control in patients with chronic periodontitis and type 2 diabetes: a meta-analysis of randomized clinical trials. J Periodontol 2013; 84(7): 958–73.

31. Teshome A, Yitayeh A. The effect of periodontal therapy on glycemic control and fasting plasma glucose level in type 2 diabetic patients: systematic review and meta-analysis. BMC Oral Health 2016; 17(1): 31.

32. Artese HPC, Foz AM, Rabelo M de S, et al. Periodontal therapy and systemic inflammation in type 2 diabetes mellitus: a meta-analysis. PLoS ONE 2015; 10(5). doi:10.1371/journal.pone.0128344.

33. Mathur LK, Manohar B, Shankarapillai R, Pandya D. Obesity and periodontitis: a clinical study. J Indian Soc Periodontol 2011; 15(3): 240–44.

34. Jagannathachary S, Kamaraj D. Obesity and periodontal disease. J Indian Soc Periodontol 2010; 14(2): 96–100.

35. Levine RS. Obesity, diabetes and periodontitis-a triangular relationship? Br Dent J 2013; 215: 35–39.

36. Engebretson S, Kocher T. Evidence that periodontal treatment improves diabetes outcomes: a systematic review and meta-analysis. J Clin Periodontol 2013; 40(Suppl 14): 153–63.

37. Graziani F, Gennai S, Solini A, Petrini M. A systematic review and meta-analysis of epidemiologic observational evidence on the effect of periodontitis on diabetes An update of the EFP-AAP review. J Clin Periodontol 2018; 45(2): 167–87.

38. Madianos PN, Koromantzos PA. An update of the evidence on the potential impact of periodontal therapy on diabetes outcomes. J Clin Periodontol 2018; 45(2): 188–95.

39. Sanz M, Ceriello A, Buysschaert M, et al. Scientific evidence on the links between periodontal diseases and diabetes: Consensus report and guidelines of the joint workshop on periodontal diseases and diabetes by the International Diabetes Federation and the European Federation of Periodontology. J Clin Periodontol 2018; 45(2): 138–49.

40. Saengtipbovorn S, Taneepanichskul S. Effectiveness of lifestyle change plus dental care program in improving glycemic and periodontal status in aging patients with diabetes: a cluster, randomized, controlled trial. Journal of Periodontology 2015; 86(4): 507–15.

41. Ahdi M, Teeuw WJ, Meeuwissen HGTA, et al. Oral health information from the dentist to the diabetologist. Eur J Intern Med 2015; 26(7): 498–503.

Attachment 13 Article ”Gene variability in immunoregulatory factors in patients with chronic periodontitis and diabetes mellitus”

Původní práce
Original Article

Genová variabilita v imunoregulačních faktorech u pacientů s chronickou parodontitidou a diabetes mellitus

ČESKÁ STOMATOLOGIE
ročník 115,
2015, 4,
s. 89–97

(Původní práce – experimentální studie)

Gene Variability in Immunoregulatory Factors in Patients with Chronic Periodontitis and Diabetes Mellitus

(Original Article – Experimental Study)

Bořilová Linhartová P.[1, 2], Valová S.[2], Kaštovský J.[1, 2], Poskerová H.[1], Vokurka J.[1], Fassmann A.[1], Izakovičová Hollá L.[1, 2]

[1]Stomatologická klinika LF MU a FN u sv. Anny, Brno
[2]Ústav patologické fyziologie LF MU, Brno

SOUHRN

Úvod a cíl studie: Genetické predispozice hrají významnou roli v etiopatogenezi jak chronické parodontitidy (CP), tak i diabetes mellitus (DM), mezi kterými existuje reciproční vztah. U obou chorob jsou za kandidátní považovány geny pro imunoregulační faktory, k nimž se řadí signální peptidy zvané cytokiny. Cílem naší studie bylo analyzovat polymorfismy ve vybraných cytokinech, konkrétně v *interleukin-1 (IL-1)* genovém klasteru a v *IL-6* genu, u pacientů s CP s/bez DM a porovnat je se zdravou populací.
Metodika: Do studie případů a kontrol (tzv. case-control study) jsme zařadili celkem 590 osob: 226 zdravých; 248 pacientů pouze s CP, 36 pacientů s CP i DM 1. typu (T1DM), 80 pacientů s CP a DM 2. typu (T2DM). Pro genotypizaci jsme využili postupů založených na polymerázové řetězové reakci (PCR) a následné restrikční analýze.
Výsledky: Frekvence alel a genotypů sledovaných polymorfismů ve vybraných cytokinech, kromě varianty v *IL-1B* genu se mezi skupinami pacientů a zdravými jedinci statisticky významně nelišily. V *IL-1* genovém klastru jsme však nalezli protektivní haplotyp u CP a haplotypy zvyšující riziko rozvoje CP u pacientů s T2DM i s T1DM. Rovněž v genu pro *IL-6* jsme prokázali signifikantní rozdíl ve frekvenci haplotypu GGG mezi pacienty s CP a zdravými jedinci.
Závěr: Naše výsledky naznačují, že na rozdíl od analýz jednotlivých polymorfismů různé haplotypy v *IL-1* genovém klasteru i v promotoru genu pro *IL-6* mohou ovlivňovat riziko rozvoje CP u pacientů s/bez DM v české populaci. Domníváme se, že stanovení haplotypů má, oproti sledování jednotlivých alelických a genotypových frekvencí, lepší výpovědní hodnotu a měla by mu být dána při genetické analýze komplexních nemocí přednost.
Klíčová slova: ***parodontitida – diabetes mellitus – genová variabilita – cytokiny – interleukiny***

SUMMARY

Introduction and aim of study: Genetic predispositions play an important role in the etiopathogenesis of periodontal diseases (CP) and diabetes mellitus (DM). Recent studies have also proven a bidirectional interrelationship between these conditions. The immunoregulatory factors, including signal peptides, so called cytokines, are considered as candidate genes for both of these diseases. The aim of our study was to analyse polymorphisms in selected cytokines, namely in the *interleukin-1 (IL-1)* gene cluster and *IL-6* gene, in patients with CP and with/without DM and compare them with the healthy population.
Methods: 590 subjects were enrolled in this case-control study: 226 healthy/non-periodontitis subjects, 248 patients only with CP, 36 patients with CP and type 1 DM (T1DM), 80 patients with CP and

ČESKÁ STOMATOLOGIE
ročník 115,
2015, 4,
s. 89–97

type 2 DM (T2DM). For genotyping methods based on polymerase chain reaction (PCR) and subsequent restriction analysis were used.
Results: The allele and genotype frequencies of the studied polymorphisms, except for a variant in the *IL-1B* gene, did not differ significantly between the groups of patients and healthy controls. However, in the *IL-1* gene cluster, a protective haplotype in CP and risk haplotypes for CP in patients with T2DM or with T1DM were found. In addition, in the *IL-6* gene, a significant difference in GGG haplotype frequencies between the groups of patients with CP and healthy controls was identified.
Conclusion: Our results suggest, that contrary to analyses of individual polymorphisms, different haplotypes in the *IL-1* gene cluster and *IL-6* gene promoter may affect the risk for CP development in patients with/without DM in Czech population. We assume that the determination of haplotypes may have a better predictive value than study of individual allele and genotype frequencies and it should be preferred in genetic analysis of complex diseases.
KEYWORDS: ***periodontitis – diabetes mellitus – gene variability – cytokines – interleukins***

Čes. Stomat., roč. 115, 2015, č.4, s. 89-97

ÚVOD A CÍLE STUDIE

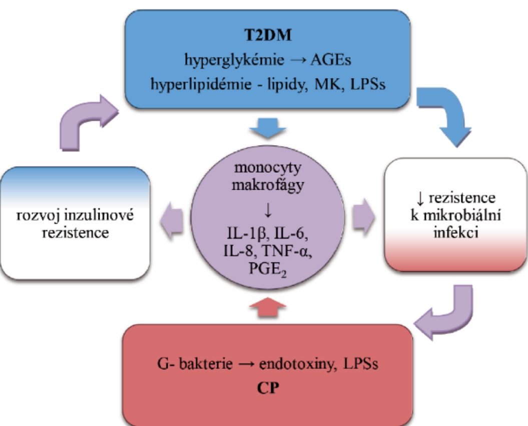
Význam vybraných cytokinů u parodontitidy a diabetes mellitus

Parodontitida je onemocnění vyvolané bakteriemi (zejména gramnegativními), při kterém dochází k rozvoji chronického zánětu, což má za následek ztrátu pojivové tkáně, alveolární kosti a nakonec i zubů. Rovněž průběh diabetes mellitus (DM), který zahrnuje skupinu metabolických onemocnění charakterizovaných hyperglykémií, je chronický a na jeho vzniku i progresi se podílí zánětlivá reakce. Mezi oběma chorobami existuje reciproční vztah, který je v poslední době intenzivně studován [4]. Kromě environmentálních faktorů (stres, kouření, obezita atd.) hraje v etiopatogenezi těchto komplexních chorob roli také genetická predispozice. Za kandidátní jsou mimo jiné považovány geny pro imunoregulační faktory, k nimž se řadí signální peptidy zvané cytokiny. Význam cytokinů, především pak interleukinu-1 (IL-1) a interleukinu-6 (IL-6), v obousměrném vztahu chronické parodontitidy (CP) a DM 2. typu (T2DM) je znázorněn na obr. 1 [28, 33].

Interleukin-1

IL-1 je pluripotentní cytokin, který patří k hlavním mediátorům zánětlivého procesu. Prozánětlivou aktivitu vykazují jeho dvě formy IL-1α a IL-1β v kontrastu s protizánětlivými schopnostmi antagonisty IL-1 receptoru (IL-1RA) [8].

U pacientů s parodontitidou byly v gingivě nalezeny zvýšené hladiny IL-1α a IL-1β [18] a významně vyšší hladiny IL-1β v gingivální tekutině vykazovali také pacienti s DM 1. typu (T1DM) [32] a pacienti s T2DM s CP ve srovnání s nediabetickými kontrolami [3, 6]. Hypotézu o vlivu hyperglykémie na stupeň zánětlivé odpovědi u parodontitidy potvrdila studie na potkaním modelu, jejíž výsledky naznačují, že DM může zvyšovat produkci IL-1β, tumor nekroti-



Obr. 1 **Význam cytokinů v obousměrném vztahu chronické parodontitidy (CP) a diabetes mellitus 2. typu (T2DM), upraveno podle [26, 31].**
Hyperglykémie charakteristická pro onemocnění T2DM je příčinným faktorem vzniku pozdních produktů glykace (AGEs), což společně s vyšší koncentrací lipidů, mastných kyselin (MK) a s lipopolysacharidy (LPSs) ovlivňuje nejen funkci buněk imunitního systému a produkci prozánětlivých molekul, ale také narušuje metabolismus tkání parodontu. V důsledku těchto dějů dochází ke snížení odolnosti vůči mikrobiální infekci vedoucí ke vzniku a rozvoji CP, kde gramnegativní (G-) bakterie uvolňují endotoxiny a LPSs a indukují odpověď monocytů a makrofágů ve formě aktivace mediátorů zánětu. Mezi ně patří tumor nekrotizující faktor alfa (TNF-α), prostaglandin E2 (PGE2) a dále celá řada cytokinů, především pak interleukin-1β (IL-1β), IL-6 a IL-8, které se mohou podílet na rozvoji inzulinové rezistence a na destrukci β-buněk pankreatu. Mezi T2DM a CP vzniká tzv. bludný kruh (circulus vitiosus); funkce cytokinů a variabilita v jejich genech tak mohou hrát významnou roli v pochopení těchto komplikovaných vztahů.

ČESKÁ STOMATOLOGIE
ročník 115,
2015, 4,
s. 89–97

zujícího faktoru-alfa (TNF-α) a lipopolysacharidů (LPSs) v parodontálních tkáních [17]. V nedávno publikované studii bylo zjištěno, že IL-1β a TNF-α mohou ovlivňovat inzulinovou rezistenci a poškození β-buněk pankreatu [27]. Protektivní role je pak přisuzována IL-1RA, jehož vyšší exprese ve slinách byla pozorována u pacientů s T2DM a zdravým parodontem [12].

Proteiny IL-1α, IL-1β a IL-1RA jsou kódovány geny *IL-1A*, *IL-1B* a *IL-1RN*, které jsou součástí *IL-1* genového klastru o velikosti 430 kb lokalizovaného na chromozomu 2q14-q21 [37]. Polymorfismy v těchto genech mohou přispět ke změnám v produkci příslušných cytokinů a jsou intenzivně zkoumány u různých zánětlivých chorob. Existují také studie u pacientů s T2DM zabývající se vztahem mezi parodontitidou a variabilitou v *IL-1* genovém klastru [11, 22, 38].

Interleukin-6

Patogeneze obou onemocnění je spojená i s vysokými hladinami IL-6 [10, 29, 39], pleiotropního cytokinu zapojujícího se do regulace imunitního systému a zánětlivých procesů, hematopoezy a metabolismu kostí. IL-6 také ovlivňuje inzulinovou rezistenci v játrech i tukové tkáni a společně s dalšími prozánětlivými cytokiny se může podílet na poškození β-buněk Langerhansových ostrůvků [21].

Jelikož míra exprese *IL-6* genu může být ovlivněna polymorfismy v jeho promotorové oblasti [41], probíhají intenzivní studie zkoumající jednonukleotidové polymorfismy (SNPs) -174G/C (rs1800795), -572G/C (rs1800796) a -597G/A (rs1800797) u pacientů s CP s/bez DM ve srovnání se zdravými kontrolami [35, 36, 42]. Nicméně dosavadní analýzy v rozličných populacích, které zkoumaly především rozdíly v alelických a genotypových frekvencích jednotlivých polymorfismů, jsou obdobně jako u studií zabývajících se variabilitou v *IL-1* genu rozporuplné.

Cíl práce

Cílem naší studie bylo analyzovat nejenom alelové a genotypové distribuce polymorfismů ve vybraných cytokinech, konkrétně IL-1α a β, antagonisty jeho receptoru a IL-6, ale zaměřili jsme se též na možnou asociaci jejich alelických kombinací (tzv. haplotypů) s CP s/bez DM.

MATERIÁL A METODY

Popis souboru a klinické vyšetření

Do studie případů a kontrol (tzv. case-control study) jsme vybrali 590 nepříbuzných osob z regionu jižní Moravy, které jsme vyšetřili v letech 2005 až 2015 na Parodontologickém oddělení Stomatologické kliniky Fakultní nemocnice u svaté Anny v Brně (H.P., J.V., A.F.). Kritéria pro zařazení do studie byla následující: věk vyšší než 30 let, přítomnost více než 16 vlastních zubů (kromě třetích molárů), pacienti nesměli podstoupit parodontologickou léčbu během uplynulých šesti měsíců anebo užívat v posledních třech měsících antibiotika. Ze studie jsme vyloučili těhotné a kojící ženy, pacienty po transplantacích orgánů, osoby se závažnými systémovými chorobami (hematologická, onkologická a autoimunitní onemocnění, infekce HIV) a pacienty dlouhodobě užívající kortikosteroidy, imunosupresiva a nesteroidní antiflogistika. Základní charakteristika pacientů i kontrolních osob je uvedena v tabulce 1.

U pacientů s CP i osob bez parodontitidy jsme odebrali celkovou a stomatologickou anamnézu, detailně klinicky vyšetřili stav chrupu a parodontu a provedli rentgenologické vyšetření. Pomocí parodontální sondy UNC-15 jsme provedli na každém zubu měření a zaznamenali hloubku parodontálních chobotů (probing pocket depth, PD) ve čtyřech lokalitách (distálně, vestibulárně, meziálně, orálně). Ve stejných lokalitách jsme měřili velikost gingiválních recesů (GR) a zapsali jsme i lokality s výraznou hyperplazií gingivy. Z těch-

Tab. 1 Demografická a klinická data pacientů s CP, CP+T1DM, CP+T2DM a kontrolních osob

Charakteristika	Kontroly N = 226	CP N = 248	CP+T1DM N = 36	CP+T2DM N = 80
Věk (průměrný věk±SD)	52,8±11,0	54,4±8,0	49,8±9,6	65,6±9,6
Pohlaví (muži/ženy)	115/111	117/131	16/20	40/40
Kouření (ne/ano, %)	72,6/27,4	71,3/29,7	77,8/22,2	87,7/12,3
Doba trvání diabetu (roky±SD)	-	-	24,7±11,6	11,2±8,7
PD (průměr v mm±SD)	1,21±0,30	3,23±0,76	3,54±0,93	3,80±1,03
GR (průměr v mm±SD)	0,16±0,28	0,72±0,53	0,87±0,77	1,09±0,78
CAL (průměr v mm±SD)	1,38±0,25	3,96±1,01	4,41±1,48	4,88±1,31

CAL = klinická ztráta úponu, CP = chronická parodontitida, GR = gingivální recesy, N = počet jedinců, PD = hloubka parodontálních chobotů, SD = směrodatná odchylka, T1DM = diabetes mellitus 1. typu, T2DM = diabetes mellitus 2. typu

ČESKÁ STOMATOLOGIE
ročník 115,
2015, 4,
s. 89–97

to údajů jsme následně vypočítali klinickou ztrátu úponu (clinical attachment loss, CAL).

Zkoumaný soubor zahrnoval:

i) 226 zdravých osob, které netrpěly CP, DM ani jinými systémovými chorobami podle výše uvedených kritérií. Osoby zařazené do kontrolní skupiny měly hodnotu CAL menší než 2 mm.

ii) 364 pacientů s generalizovanou CP, kteří splňovali diagnostická kritéria definovaná podle American Academy of Periodontology (AAP) [2]. Mezi kritéria pro zařazení pacientů do této skupiny patřilo: minimálně 30 % postižených zubů a CAL ≥ 2 mm. Tyto pacienty jsme dále rozdělili do tří podskupin v závislosti na přítomnosti onemocnění a typu DM:

1. 248 pacientů bez DM a jiných systémových onemocnění,
2. 36 pacientů s T1DM,
3. 80 pacientů s T2DM.

U pacientů s DM jsme zaznamenali hladiny glykémie, glykovaného hemoglobinu (HbA1c) a lipidů (celkový cholesterol, HDL, LDL a triacylglyceroly), viz tab. 2, dále farmakoterapii a informace o komplikacích spojených s DM podle zdravotní dokumentace z diabetologických ordinací. Do studie jsme zařadili jen ty osoby s DM, u kterých onemocnění trvalo déle než tři roky.

Studie byla schválena Etickou komisí Lékařské fakulty Masarykovy univerzity a Fakultní nemocnice u svaté Anny v Brně. Písemný informovaný souhlas podepsaly všechny osoby před zařazením do studie v souladu s Helsinskou deklarací.

Izolace DNA a genotypizace

Izolaci DNA a stanovení genotypů pro SNPs v *IL-1* genovém klasteru: *IL-1A* -889C/T (rs1800587), *IL-1B* +3953C/T (rs1143634) a variabilního počtu tandemových repetic (VNTR polymorfismus) ve 2. intronu genu pro *IL-1RN* jsme provedli podle již dříve publikovaných protokolů [16] (P.B.L., J.K.).

Analýza polymorfismů v promotoru *IL-6* genu -174G/C (rs1800795), -572G/C (rs1800796) a -597G/A (rs1800797) byla založena na principu polymerázové řetězové reakce (PCR) s následnou restrikcí vybranými enzymy; postupovali jsme podle našich již dříve zveřejněných protokolů [15] (S.V.).

Všechny genetické analýzy byly provedeny bez znalosti osobních a klinických údajů vyšetřovaných osob. U 10 % vzorků DNA jsme stanovení genotypů opakovali pro ověření správnosti analýzy.

Statistické hodnocení

Pro hodnocení jsme použili standardní metody deskriptivní statistiky; počítali jsme absolutní a relativní frekvence pro kategoriální proměnné a průměr se směrodatnou odchylkou pro kvantitativní proměnné. Vzhledem k normálnímu rozložení naměřených hodnot jsme stav parodontu i laboratorní nálezy hodnotili pomocí analýzy variance (ANOVA). Významnost rozdílů v alelických frekvencích mezi jednotlivými skupinami jsme počítali Fisherovým exaktním testem. Signifikance odchylek od Hardy-Weinbergova ekvilibria pro každý polymorfismus a rozdíly ve frekvencích genotypů jsme testovali pomocí χ^2-testu. Haplotypovou analýzu jsme provedli s využitím programu SNP analyzer v. 2.0. (http://snp.istech21.com/snpanalyzer/2.0/); hodnotu $p < 0,05$ jsme považovali za statisticky významnou (L.I.H.).

VÝSLEDKY

Klinické vyšetření

Postižení parodontu u pacientů s CP s/bez diabetu a u zdravých osob jsme hodnotili pomocí PD, GR a CAL, viz tab. 1. U všech skupin pacientů byly hodnoty PD, GR a CAL významně vyšší při porovnání se zdravými kontrolami ($p < 0,01$). Statisticky významný rozdíl jsme pozorovali i při srovnání pacientů s CP a CP+T2DM ($p < 0,01$). U nemocných s CP a diabetem obou typů jsme hodnotili také koncentraci glukózy v krvi, HbA1c a lipidů (celkového cholesterolu, HDL, LDL a triacylglycerolů) (tab. 2). Ačkoli se glykémie nalačno nelišila mezi skupinou pacientů s CP+T1DM a CP+T2DM, hladina HbA1c byla významně vyšší u pacientů s T1DM. Tito pacienti měli také vyšší koncentraci HDL cholesterolu ($p = 0,01$) a nižší průměrnou koncentraci triacylglycerolů ($p < 0,01$) při porovnání se skupinou pacientů s CP+T2DM.

Tab. 2 Koncentrace glukózy v krvi (glykémie), glykovaného hemoglobinu a lipidů u pacientů s CP+T1DM a CP+T2DM

Charakteristika	CP+T1DM	CP+T2DM	p
glykémie (mmol/l±SD)	7,34±2,34	7,57±2,13	0,61
HbA1c (mmol/mol±SD)	69,36±11,56	58,26±15,66	0,001*
celkový cholesterol (mmol/l±SD)	4,78±0,72	4,77±1,01	0,94
HDL (mmol/l±SD)	1,65±0,39	1,33±0,63	0,01*
LDL (mmol/l±SD)	2,70±0,64	2,81±92	0,56
TAG (mmol/l±SD)	1,01±0,64	1,96±1,14	0,001*

CP = chronická parodontitida, HbA1c = glykovaný hemoglobin, p = p-hodnota, SD = směrodatná odchylka, TAG = triacylglyceroly, T1DM = diabetes mellitus 1. typu, T2DM = diabetes mellitus 2. typu, *p < 0,05

Interleukin-1

Neprokázali jsme statisticky významnou asociaci alelických ani genotypových frekvencí *IL-*

ČESKÁ STOMATOLOGIE
ročník 115,
2015, 4,
s. 89–97

Tab. 3 Genotypové a alelové frekvence polymorfismu v *IL-1B* +3953C/T (rs1143634) u pacientů s CP, CP+T1DM, CP+ T2DM a u kontrol

Genotypy Alely	Kontroly	CP	OR (95% CI)	CP+T1DM	OR (95% CI)	CP+T2DM	OR (95% CI)
IL-1B +3953 C/T	N (%)	N (%)		N (%)		N (%)	
CC	115 (50,9)	156 (62,9)	1,00	18 (50,0)	1,00	48 (60,0)	1,00
CT	95 (42,0)	80 (32,3)	0,62 (0,42-0,91)	16 (44,4)	1,08 (0,52-2,22)	26 (32,5)	0,66 (0,38-1,14)
TT	16 (7,1)	12 (4,8)	0,55 (0,25-1,21)	2 (5,6)	0,80 (0,17-3,77)	6 (7,5)	0,90 (0,33-2,43)
C	325 (71,9)	392 (79,0)	1,00	52 (72,2)	1,00	122 (76,3)	1,00
T	127 (28,1)	104 (21,0)*	0,68 (0,50-0,91)	20 (27,8)	0,98 (0,57-1,71)	38 (23,8)	0,80 (0,52-1,21)

CI = interval spolehlivosti, CP = chronická parodontitida, *IL-1* = interleukin-1, OR = poměr šancí, T1DM = diabetes mellitus 1. typu, T2DM = diabetes mellitus 2. typu *p < 0,05

1A -889C/T (rs1800587) SNP a VNTR ve 2. intronu *IL-1RN*, ale nalezli jsme rozdíly ve frekvencích alel *IL-1B* +3953C/T mezi zdravými kontrolami a pacienty s CP (p < 0,02) (tab. 3). Také frekvence tzv. „double genotypu“ složeného z *IL-1A* -889C/T a *IL-1B* +3953C/T SNPs, který byl dříve označovaný jako „s parodontitidou asociovaný genotyp“, nebyla mezi jednotlivými skupinami statisticky významně rozdílná.

Pro haplotypovou analýzu, při které jsou sledovány frekvence kombinací jednotlivých typů alel zkoumaných polymorfismů, jsme alely s více než dvěma repeticemi označili jako *IL-1RN**L (dlouhá alela) a alelu se dvěma repeticemi jako *IL-1RN**S (krátká alela) [16]. Výsledky haplotypové analýzy ukazují, že s rizikem CP je podle aditivního modelu vysoce signifikantně asociován haplotyp TTL (p < 0,01, OR = 0,64, 95% CI = 0,465–0,890). CCL haplotyp jsme shledali rizikový (p < 0,05, OR = 1,94, 95% CI = 1,068–3,506) u pacientů s CP a T2DM v recesivním modelu, stejně tak jako CTL haplotyp (p < 0,05, OR = 3,03, 95% CI = 1,081–8,483) u pacientů s CP a T1DM ve srovnání se zdravými osobami podle dominantního modelu (tab. 4).

Interleukin-6

Ani v případě sledovaných SNPs v promotoru *IL-6* genu jsme nenašli statisticky významný rozdíl ve frekvencích alel a genotypů mezi pacienty s CP a zdravými jedinci. Analýzou haplotypů jsme prokázali signifikantní rozdíl ve frekvenci haplotypu GGG mezi skupinou pacientů s CP a kontrolami (p < 0,03, OR = 1,679, 95% CI = 1,050–2,685) podle recesivního modelu. Nepozorovali jsme ale statisticky významné rozdíly ve frekvencích haplotypů u pacientů s CP v kombinaci s T1DM nebo T2DM v porovnání se zdravými jedinci (tab. 5).

DISKUSE

Klinické vyšetření

Souvislosti mezi diabetem a onemocněním parodontu jsou známé již řadu let. Většina autorů se shoduje v tom, že diabetes zvyšuje prevalenci,

Tab. 4 Frekvence *IL-1* haplotypů u pacientů s CP, CP+T1DM, CP+T2DM a u kontrol

IL-1A -889C/T	*IL-1B* +3953C/T	*IL-1RN* (VNTR)§	Kontroly	CP	CP+T1DM	CP+T2DM
C	C	L	0,4319	0,4857	0,4749	0,5159*
C	C	S	0,2153	0,2185	0,1861	0,1878
T	T	L	0,2146	0,1342*	0,1661	0,1676
T	C	L	0,0497	0,0590	0,0339	0,0322
C	T	L	0,0318	0,0348	0,0890*	0,0156
T	C	S	0,0222	0,0271	0,0273	0,0266
T	T	S	0,0211	0,0378	0,0227	0,0361
C	T	S	0,0136	0,0029	-	0,0182

CP = chronická parodontitida, *IL-1* = interleukin-1, T1DM = diabetes mellitus 1. typu, T2DM = diabetes mellitus 2. typu

§Pro haplotypovou analýzu byly alely s více než dvěmi repeticemi sloučeny do skupiny s názvem *IL-1RN*L* („long“ alela), alela s dvěmi repeticemi byly označena jako *IL-1RN*S* („short“alela).

Haplotypy jsou seřazeny sestupně dle frekvence haplotypů u kontrol.

***p < 0,05 vs. kontroly**

ČESKÁ STOMATOLOGIE
ročník 115,
2015, 4,
s. 89–97

Tab. 5 Frekvence *IL-6* haplotypů u pacientů s CP, CP+T1DM, CP+T2DM a u kontrol

IL-6 -174G/C	*IL-6 -572G/C*	*IL-6 -597G/A*	Kontroly	CP	CP+T1DM	CP+T2DM
G	G	G	0,4596	0,5228*	0,5000	0,5088
C	G	A	0,4145	0,4214	0,3649	0,4222
G	C	G	0,0645	0,0423	0,1216	0,0498
G	G	A	0,0340	0	0	0
C	G	G	0,0230	0,0135	0,0135	0,0193
C	C	A	0,0046	0	0	0

CP = chronická parodontitida, *IL-6 = interleukin-6*, T1DM = diabetes mellitus 1. typu, T2DM = diabetes mellitus 2. typu

Haplotypy jsou seřazeny sestupně podle frekvence haplotypů u kontrol.

*$p < 0,05$ vs. kontroly

Pozn. Při analýze polymorfismů v *IL-6* genu jsme sledovali alelické, genotypové a haplotypové frekvence pouze u 181 zdravých osob, 223 pacientů s CP, 36 pacientů s CP a T1DM a 78 pacientů s CP a T2DM.

incidenci, progresi a závažnost parodontitidy [13, 40]. Podle předpokladů byly hodnoty PD, GR i CAL statisticky významně vyšší ve všech skupinách pacientů ve srovnání se zdravými kontrolami. Nejhorší stav parodontu (s největší klinickou ztrátou úponu) jsme pozorovali u pacientů s CP+T2DM, a to i přes fakt, že v této skupině bylo nejméně kuřáků a pacienti trpěli DM méně než polovinu let ve srovnání s pacienty s T1DM. Pacienti s T2DM však byli o více než patnáct let starší než osoby v ostatních skupinách. Zajímavým nálezem byly stejné koncentrace glykémie u pacientů s T1DM a T2DM, avšak výrazně vyšší hodnoty HbA1c u pacientů s T1DM. Zvýšená koncentrace triacylglycerolů a snížená hladina HDL cholesterolu u pacientů s T2DM odpovídá typickému obrazu „diabetické dyslipidémie“ [7].

Interleukin-1

Asociaci *IL-1A* -889C/T (rs1800587) polymorfismu u pacientů s CP s/bez DM v české populaci jsme neprokázali, což je v kontrastu s výsledky metaanalýzy Karimbuxe a kol. [19]. Dosud nebyla publikována práce zabývající se tímto polymorfismem u pacientů s T1DM, ale u pacientů s T2DM byla varianta v pozici -889 *IL-1A*, konkrétně alela T, asociována s vyšším rizikem T2DM u mužů, zatímco u žen měla tato minoritní alela opačný, tedy protektivní, ale rovněž signifikantní efekt [24].

Z výsledků naší studie vyplývá, že polymorfismus *IL-1B* +3953C/T (rs1143634) může hrát roli v etiopatogenezi CP v české populaci, kdy se alela T jeví jako protektivní. Tato pozorování podporují námi dříve zjištěnou asociaci C alely *IL-1B* +3953 polymorfismu s rizikem rozvoje CP [14]. Nicméně funkční význam SNPs IL-1B +3953C/T varianty v korelaci s produkcí IL-1β není ještě dostatečně zmapován a dosavadní studie poskytují kontroverzní výsledky [25]. U pacientů s CP a DM jsme významný vztah ke studovanému *IL-1B* polymorfismu neprokázali, ačkoli Krikovsky a kol. [20] považují T alelu u SNP *IL-1B* +3953 (označovanou také jako +3954) za rizikovou u dětí s T1DM. Alela T v pozici +3953 *IL-1B* genu byla také již dříve spojena s vyšší koncentrací glukózy v krvi u pacientů s DM [23] a Guzman a kol. [11] nalezli marginální vztah mezi polymorfismem v pozici +3953C/T *IL-1B* genu a výskytem parodontitidy v diabetické populaci. Struch a kol. [38] ve své práci uvádějí, že pacienti s T2DM mají zvýšené riziko CP, které dále narůstá při určité kombinaci variant *IL-1A/1B* genotypu. Nicméně v české populaci jsme statisticky významný rozdíl ve frekvenci tzv. „double genotypu“ složeného z *IL-1A* -889C/T a *IL-1B* +3953C/T SNPs mezi skupinami pacientů ve srovnání s kontrolami nenašli.

Vzhledem k tomu, že prozánětlivý potenciál IL-1α a IL-1β je regulován endogenními inhibitory, jakými je např. antagonista IL-1 receptoru, také variabilita v jeho genu by mohla hrát důležitou roli v etiopatogenezi daných onemocnění. V naší studii jsme však nezjistili významný vztah mezi *IL-1RN* (VNTR, 86 bp v intronu 2) variantami u pacientů s CP s/bez DM, což je v souladu s výsledky metaanalýzy od Ding a kol. [9], kde *IL-1RN* VNTR byl asociován pouze s agresivní, ne však s chronickou parodontitidou. V egyptské populaci byla frekvence krátké alely *IL-1RN**S a genotypu tvořeného kombinací dvou krátkých alel (tj. *IL-1RNSS*) nižší u pacientů s T1DM ve srovnání se zdravými kontrolami [34] a v indické populaci tuto krátkou alelu společně s *IL-1RNSS* genotypem asociovali s rizikem rozvoje T2DM [1, 5].

S našimi výsledky korespondují pozorování v chilské populaci, kdy López a kol. [22] neprokázali významné rozdíly v alelických ani genotypových frekvencích polymorfismů *IL-1A* -889C/T, *IL-1B* +3953C/T a *IL-1RN* VNTR mezi pacienty s CP s/bez T2DM, na

ČESKÁ STOMATOLOGIE
ročník 115,
2015, 4,
s. 89–97

druhou stranu však potvrdili asociaci TT genotypů obou polymorfismů *IL-1A* -889C/T i *IL-1B* +3953C/T s výskytem CP. Domníváme se, že diskrepance mezi výsledky různých studií je zapříčiněna nejenom populačními rozdíly, ale především dosud nedostatečnou komplexností genetického vyšetření. Proto se přikláníme k haplotypové analýze, která, na rozdíl od analýz jednotlivých genových variant, poskytuje ucelenější pohled na vztah polymorfismů v *IL-1* genovém klastru k CP a DM.

Při srovnání haplotypových frekvencí se haplotyp TTL, složený z *IL-1A* -889C/T, *IL-1B* +3953C/T a *IL-1RNS/L* VNTR, zdá být protektivní k rozvoji CP. Předpokládáme, že dříve popsaný „ochranný“ vliv T alely *IL-1B* +3953C/T polymorfismu na rozvoj CP v naší populaci, se uplatňuje také u TTL haplotypu u stejné skupiny pacientů. Určitou roli rovněž může hrát počet repetic v *IL-1RN* genu, neboť při porovnání frekvencí TTL a TTS haplotypů u pacientů s CP a zdravých kontrol je ten s krátkou alelou (2 repetice) v *IL-1RN* VNTR spíše rizikový pro rozvoj CP. U pacientů s kombinací CP a T1DM můžeme označit jako rizikový haplotyp CTL, zatímco CCL haplotyp zvyšuje riziko vzniku CP a T2DM. Z důvodu rozdílné etiopatogeneze onemocnění T1DM a T2DM nejsou výsledky haplotypové analýzy překvapující.

Interleukin-6

Také polymorfismy v promotoru genu *IL-6* jsou kvůli jejich možnému vlivu na míru exprese tohoto cytokinu [41] intenzivně zkoumány. Metaanalýza několika studií potvrdila asociaci *IL-6* -174G/C (rs1800795) polymorfismu s CP pouze u brazilské populace, zatímco alela G *IL-6* -572G/C (rs1800796) varianty byla spojena s rizikem rozvoje CP bez závislosti na etnicitě populace [36]. V naší studii jsme neprokázali vztah jednotlivých alel či genotypů SNPs *IL-6* -174G/C (rs1800795), -572G/C (rs1800796) a -597G/A (rs1800797) k rozvoji CP u pacientů s/bez DM. V čínské populaci byla protektivním faktorem pro CP s T2DM alela C polymorfismu *IL-6* -572G/C (rs1800796) [42] a u indické populace alela A polymorfismu *IL-6* -597G/A (rs1800797) [35]. Rozdílné výsledky jednotlivých studií mohou být dány jednak odlišnou frekvencí polymorfismů v různých populacích a také tím, že se nebere v potaz možnost vzájemného ovlivňování jednotlivých SNPs. Polymorfismy totiž nemusí při transkripci *IL-6* genu působit aditivně, ale spíše skrze složité interakce definované haplotypem [41].

Ačkoliv jsme u skupin pacientů s kombinací obou onemocnění (tj. s CP s T1DM/T2DM) nepozorovali signifikantní rozdíly ve frekvencích *IL-6* haplotypů, haplotyp G(-174)/G(-572)/G(-597) byl v naší studii spojen s rizikem rozvoje CP. Jelikož je patogeneze parodontitidy spojena se zvýšenou hladinou IL-6 [39], mohl by být haplotyp GGG v porovnání s ostatními haplotypy více transkripčně aktivní. Tento předpoklad je v souladu s experimentem, při kterém konstrukty s haplotypem GGG transfekované do buněk a následně stimulované IL-1 vykazovaly oproti ostatním konstruktům s odlišnými haplotypy vyšší transkripci [41]. Výsledky pokusů zabývající se u zdravých jedinců s různými haplotypy koncentrací IL-6 v plné krvi po stimulaci LPSs jsou protichůdné. Müller-Steinhardt a kol. [26] naměřili při tomto experimentu u homozygotních nositelů genotypu GGG v porovnání s ostatními genotypy nižší množství produkovaného IL-6. V kontrastu s touto studií je práce od Rivera-Chavez a kol. [31], kteří pozorovali u homozygotů GGG ve srovnání s ostatními genotypy vyšší hladinu IL-6, což je v souladu s našimi závěry. Rozporuplné výsledky výše zmíněných dvou studií mohou být způsobeny použitím různých protokolů pro inkubaci, stimulaci plné krve, měření koncentrace IL-6 či existencí jiných než zkoumaných SNPs podílejících se na expresi *IL-6*. Dalším limitem těchto pokusů je jejich *ex vivo* provedení, tudíž se mimo jiné nezohledňuje vliv polymorfismu Asp358Ala (+48892A/C, rs2228145) v genu pro receptor *interleukinu-6* (*IL-6R*), který *in vivo* zvyšuje hladinu IL-6 v plazmě [30].

Limitací naší studie je počet pacientů s CP a současně s DM, což je důsledek relativně přísných kritérií, která pro zařazení do této podskupiny dodržujeme. Nízké počty zahrnutých pacientů v těchto skupinách, mohou být důvodem negativních výsledků z důvodu nízké „síly testu“. Na druhou stranu jsou jedinci, zahrnutí do skupin pacientů a zdravých kontrol, velmi dobře anamnesticky a klinicky vyšetřeni, studovaná kohorta je etnicky homogenní a velikost souboru pacientů s CP bez DM a osob bez parodontitidy je v porovnání s jiným studiemi tohoto charakteru výrazně větší.

ZÁVĚR

Závěrem lze říci, že polymorfismus +3953C/T (rs1143634) v *IL-1B* genu může ovlivňovat náchylnost k CP v české populaci, avšak pro ucelenější pochopení vlivu variability ve studovaných genech vybraných cytokinů je vhodnější haplotypová analýza. Určité haplotypy v *IL-1* a *IL-6* genech lze považovat za rizikové, či naopak protektivní při rozvoji daných onemocnění. Domníváme se, že výsledky naší práce mohou být využity k lepšímu porozumění vztahu mezi CP a DM na molekulární úrovni a v budoucnu přispějí ke zlepšení diagnostiky, případně terapie těchto komplexních chorob.

ČESKÁ STOMATOLOGIE
ročník 115, 2015, 4, s. 89–97

LITERATURA

1. **Achyut, B. R., Srivastava, A., Bhattacharya, S., Mittal, B.:** Genetic association of interleukin 1beta (-511C/T) and interleukin-1 receptor antagonist (86 bp repeat) polymorphisms with type 2 diabetes mellitus in North Indians. Clin. Chim. Acta, roč. 377, 2007, č. 1–2, s. 163–169.
2. **Armitage, G. C.:** Development of a classification system for periodontal diseases and conditions. Ann. Periodontol., roč. 4, 1999, č. 1, s. 1–6.
3. **Atieh, M. A., Faggion, C. M., Seymour, G. J.:** Cytokines in patients with type 2 diabetes and chronic periodontitis: A systematic review and meta-analysis. Diabetes Res. Clin. Pract., roč. 104, 2014, č. 2, s. 38–45.
4. **Bascones-Martínez, A., González-Febles, J., Sanz-Esporrín, J.:** Diabetes and periodontal disease. Review of the literature. Am. J. Dent., roč. 27, 2014, č. 2, s. 63–67.
5. **Bid, H. K., Konwar, R., Agrawal, C. G., Banerjee, M.:** Association of IL-4 and IL-1RN (receptor antagonist) gene variants and the risk of type 2 diabetes mellitus: a study in the north Indian population. Indian J. Med. Sci., roč. 62, 2008, č. 7, s. 259–266.
6. **Bulut, U., Develioglu, H., Taner, I. L., Berker, E.:** Interleukin-1 beta levels in gingival crevicular fluid in type 2 diabetes mellitus and adult periodontitis. J. Oral Sci., roč. 43, 2001, č. 3, s. 171 –177.
7. **Češka, R.:** Dyslipidémie u osob s diabetem 2. typu a její léčba. Kap. Kardiol., roč. 3, 2011, č. 3, s. 97–101.
8. **Dinarello, C. A.:** The IL-1 family and inflammatory diseases. Clin. Exp. Rheumatol., roč. 20, 2002, č. 5, s. 1–13.
9. **Ding, C., Zhao, L., Sun, Y., Li, L., Xu, Y.:** Interleukin-1 receptor antagonist polymorphism (rs2234663) and periodontitis susceptibility: a meta-analysis. Arch. Oral Biol., roč. 57, 2012, č. 6, s. 585–593.
10. **Erbağci, A. B., Tarakçioğlu, M., Coşkun, Y., Sivasli, E., Sibel Namiduru, E.:** Mediators of inflammation in children with type I diabetes mellitus: cytokines in type I diabetic children. Clin. Biochem., roč. 34, 2001, č. 8, s. 645–650.
11. **Guzman, S., Karima, M., Wang, H. Y., Van Dyke, T. E.:** Association between interleukin-1 genotype and periodontal disease in a diabetic population. J. Periodontol., roč. 74, 2003, č. 8, s. 1183–1190.
12. **Chan. H. H., Rahim, Z. H., Jessie, K., Hashim, O. H., Taiyeb-Ali, T. B.:** Salivary proteins associated with periodontitis in patients with type 2 diabetes mellitus. Int. J. Mol. Sci., roč. 13, 2012, č. 4, s. 4642–4654.
13. **Chapple, I. L. C., Genco, R.:** Diabetes and periodontal diseases: consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases. J. Clin. Periodontol., roč. 40, 2013, Suppl. 14, s. 106–112.
14. **Izakovičová Hollá, L., Dvořáková, N., Halaba, T., Znojil, V., Vácha, J., Vaněk, J.:** Analýza polymorfismů v genech pro interleukin-1 (IL-1) u českých pacientů s chronickou parodontitidou. Čes. Stomat., roč. 104, 2004, č. 2, s. 48–56.
15. **Izakovičová Hollá, L., Fassmann, A., Stejskalová, A., Znojil, V., Vaněk, J., Vácha, J.:** Analysis of the interleukin-6 gene promoter polymorphisms in Czech patients with chronic periodontitis. J. Periodontol., roč. 75, 2004, č. 1, s. 30–36.
16. **Izakovičová Hollá, L., Bořilová Linhartová, P., Hrdličková, B., Marek, F., Dolina, J., Řihák, V., Kala. Z.:** Haplotypes of the IL-1 gene cluster are associated with gastroesophageal reflux disease and Barrett's esophagus. Hum. Immunol., roč. 74, 2013, č. 9, s. 1161–1169.
17. **Jiang, Z. L., Cui, Y. Q., Gao, R., Li, Y., Fu, Z. C., Zhang, B., Guan, C. C.:** Study of TNF-α, IL-1β and LPS levels in the gingival crevicular fluid of a rat model of diabetes mellitus and periodontitis. Dis. Markers, roč. 34, 2013, č. 5, s. 295–304.
18. **Kamagata, Y., Miyasaka, N., Inoue, H., Hashimoto, J., Iida, M.:** Study of cytokine production in inflamed human gingival tissues in periodontitis. Interleukin-1 (IL-1 alpha, beta) and tumor necrosis factor (TNF alpha). Nihon Shishubyo Gakkai Kaishi, roč. 31, 1989, č. 3, s. 843–848.
19. **Karimbux, N. Y., Saraiya, V. M., Elangovan, S., Allareddy, V., Kinnunen, T., Kornman, K. S., Duff, G. W.:** Interleukin 1 gene polymorphisms and chronic periodontitis in adult whites: a systematic review and meta-analysis. J. Periodontol., roč. 83, 2012, č. 11, s. 1407–1419.
20. **Krikovsky, D., Vásárhelyi, B., Treszl, A., Körner, A., Tordai, A., Tulassay, T., Madácsy, L.:** Genetic polymorphism of interleukin-1beta is associated with risk of type 1 diabetes mellitus in children. Eur. J. Pediatr., roč. 161, 2002, č. 9, s. 507–508.
21. **Kristiansen, O. P., Mandrup-Poulsen, T.:** Interleukin-6 and diabetes: the good, the bad, or the indifferent? Diabetes, roč. 54, 2005, s. 114–124.
22. **López, N. J., Venezuela, C. Y., Jara, L.:** Interleukin-1 gene cluster polymorphisms associated with periodontal disease in type 2 diabetes. J. Periodontol., roč. 80, 2009, č. 10, s. 1590–1598.
23. **Luotola, K., Pääkkönen, R., Alanne, M., Lanki, T., Moilanen, L., Surakka, I., Pietilä, A., Kähönen, M., Nieminen, M. S., Kesäniemi, Y. A., Peters, A., Jula, A., Perola, M., Salomaa, V., Health 2000 AIRGENE Study Groups:** Association of variation in the interleukin-1 gene family with diabetes and glucose homeostasis. J. Clin. Endocrinol. Metab., roč. 94, 2009, č. 11, s. 4575–4583.
24. **Luotola, K., Pietilä, A., Zeller, T., Moilanen, L., Kähönen, M., Nieminen, M. S., Kesäniemi, Y. A., Blankenberg, S., Jula, A., Perola, M., Salomaa, V., Health 2000 Study, FINRISK97 Study.** Association between interleukin-1 (IL-1) gene variations of IL-1 receptor antagonist and the development type 2 diabetes. J. Intern. Med., roč. 269, 2011, č. 3, s. 322–332.
25. **Mark, L. L., Haffajee, A. D., Socransky, S. S., Kent, R. L., Guerrero, D., Kornman, K., Newman, M., Stashenko, P.:** Effect of the interleukin-1 genotype on monocyte IL-1beta expression in subjects with adult periodontitis. J. Periodontal Res., roč. 35, 2000, č. 8, s. 172–177.
26. **Müller-Steinhardt, M., Ebel, B., Härtel, C.:** The impact of interleukin-6 promoter -597/-572/-174 genotype on interleukin-6 production after lipopolysaccharide stimulation. Clin. Exp. Immunol., roč. 147, 2007, č. 2, s. 339–345.
27. **Odegaard, J. I., Chawla, A.:** Connecting type 1 and type 2 diabetes through innate immunity. Cold Spring Harb. Perspect. Med., roč. 2, 2012, č. 3, a. 007724. doi: 10.1101/cshperspect.a007724.
28. **Poskerová, H., Bořilová Linhartová, P., Vokurka, J., Fassmann, A., Izakovičová Hollá, L.:** Diabetes a orální zdraví. Čes. Stomat., roč. 114, 2014, č. 5, s. 75–86.
29. **Pradhan, A. D., Manson, J. E., Rifai, N., Buring, J. E., Ridker, P. M.:** C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. JAMA, roč. 286, 2001, č. 3. s. 327–334.
30. **Rafiq, S., Frayling, T. M., Murray, A., Hurst, A., Stevens, K., Weedon, M. N., Henley, W., Ferrucci, L., Bandinelli, S., Corsi, A.-M., Guralnik, J. M., Melzer, D.:** A common variant of the interleukin 6 receptor (IL-6r) gene increases IL-6r and IL-6 levels, without other inflammatory effects. Genes Immun., roč. 8, 2007, č. 7, s. 552–559.
31. **Rivera-Chavez, F. A., Peters-Hybki, D. L., Barber, R. C., O'Keefe, G. E.:** Interleukin-6 promoter haplotypes and interleukin-6 cytokine responses. Shock (Augusta, Ga.), roč. 20, 2003, č. 3, s. 218–223.

ČESKÁ STOMATOLOGIE
ročník 115,
2015, 4,
s. 89–97

32. **Salvi, G. E., Beck, J. D., Offenbacher, S.:** PGE2, IL-1 beta, and TNF-alpha responses in diabetics as modifiers of periodontal disease expression. Ann. Periodontol., roč. 3, 1998, č. 1, s. 40–50.
33. **Santos Tunes, R., Foss-Freitas, M. C., Nogueira-Filho Gda, R.:** Impact of periodontitis on the diabetes-related inflammatory status. J. Can. Dent. Assoc., roč. 76, 2010, s. 35.
34. **Settin, A., Ismail, A., El-Magd, M. A., El-Baz, R., Kazamel, A.:** Gene polymorphisms of TNF-alpha-308 (G/A), IL-10(-1082) (G/A), IL-6(-174) (G/C) and IL-1Ra (VNTR) in Egyptian cases with type 1 diabetes mellitus. Autoimmunity, roč. 42, 2009, č. 1, s. 50–55.
35. **Sharma, N., Joseph, R., Arun, R., Chandni, R., Srinivas, K. L., Banerjee, M.:** Cytokine gene polymorphism (interleukin-1β +3954, Interleukin-6 [-597/-174] and tumor necrosis factor-α -308) in chronic periodontitis with and without type 2 diabetes mellitus. Indian J. Dent. Res., roč. 25, 2014, č. 3, s. 375–380.
36. **Song, G. G., Choi, S. J., Ji, J. D., Lee, Y. H.:** Association between tumor necrosis factor-α promoter -308 A/G, -238 A/G, interleukin-6 -174 G/C and -572 G/C polymorphisms and periodontal disease: a meta-analysis. Mol. Biol. Rep., roč. 40, 2013, č. 8, s. 5191–5203.
37. **Steinkasserer. A., Spurr, N. K., Cox, S., Jeggo, P., Sim, R. B.:** The human IL-1 receptor antagonist gene (IL1RN) maps to chromosome 2q14-q21, in the region of the IL-1 alpha and IL-1 beta loci. Genomics, roč. 13, 1992, č. 3, s. 654–657.
38. **Struch, F., Dau, M., Schwahn, C., Biffar, R., Kocher, T., Meisel, P.:** Interleukin-1 gene polymorphism, diabetes, and periodontitis: results from the Study of Health in Pomerania (SHIP). J. Periodontol., roč. 79, 2008, č. 3, s. 501–507.
39. **Takahashi, K., Takashiba, S., Nagai, A., Takigawa, M., Myoukai, F., Kurihara, H., Murayama, Y.:** Assessment of interleukin-6 in the pathogenesis of periodontal disease. J. Periodontol., roč. 65, 1994, č. 2, s. 147–153.
40. **Taylor, J. J., Preshaw, P. M., Lalla, E.:** A review of the evidence for pathogenic mechanisms that may link periodontitis and diabetes. J. Clin. Periodontol., roč. 40, 2013, Suppl. 14, s. 113–134.
41. **Terry, C. F., Loukaci, V., Green, F. R.:** Cooperative influence of genetic polymorphisms on interleukin 6 transcriptional regulation. J. Biol. Chem., roč. 275, 2000, č. 24, s. 18138–18144.
42. **Xiao, L. M., Yan, Y. X., Xie, C. J., Fan, W. H., Xuan, D. Y., Wang, C. X., Chen, L., Sun, S. Y., Xie, B.Y., Zhang, J. C.:** Association among interleukin-6 gene polymorphism, diabetes and periodontitis in a Chinese population. Oral Dis., roč. 15, 2009, č. 8, s. 547–553.

Tato studie byla podpořena granty IGA NT11405-6, GA ČR GB14-37368G, a projektem MUNI/A/1359/2014.

Mgr. Petra Bořilová Linhartová, Ph.D.
Ústav patologické fyziologie LF MU
Kamenice 5
625 00 Brno
e-mail: plinhart@med.muni.cz