

Pro-inflammatory cytokines in saliva of adolescents with dental caries disease

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Abstract

Introduction and Objective: Dental caries is an inflammatory disease with multifactorial etiology. The presented study was conducted to test the hypothesis that the elevation of salivary cytokines – interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor (TNF- α) is changed in dental caries patients. IL-6, IL-8 and TNF- α are particularly relevant to inflammation, one of the very first responses of the host to a pathological insult.

Materials and Methods: Whole saliva from 26 patients with dental caries, as well as 10 healthy persons, was investigated for the presence of IL-6, IL-8 and TNF- α by enzyme immunoassay – ELISA.

Results: The results showed that an elevation of IL-6, IL-8 and TNF- α in unstimulated whole saliva in subjects with dental caries, compared with controls, increased and was statistically significant in all cases ($p < 0.05$). The study also show a positive correlation between TNF- α and IL-8.

Conclusions: These data suggest links between the production of tumour necrosis factor (TNF- α), interleukin-6 (IL-6), interleukin-8 (IL-8) in saliva and dental caries disease.

Key words

dental caries, saliva, interleukin-6, interleukin-8, tumour necrosis factor α , inflammation

INTRODUCTION

Dental caries is an infectious disease with multifactorial etiology [1]. A large number of research studies have been carried out to discover the cause of this disease. The process of dental caries is now well understood and is determined by a dynamic balance between pathological factors (acidogenic bacteria, reduced salivary function) and protective factors (proteins, fluoride, calcium, phosphate) [2]. It is also well known that saliva secretion and salivary components are important for dental health. Inorganic and organic components in saliva may influence the colonization and elimination of microorganisms from the oral cavity [3, 4]. Bacteria colonize the oral cavity and lead to the process of inflammation. These lesions induce both innate and adaptive immune responses by the host [2]. The predominant cell types within periapical lesions are neutrophils, macrophages, T- and B-lymphocytes, mast cells, osteoclasts, osteoblasts, and fibroblasts. These cells express a large number of proinflammatory cytokines, including interleukin IL-6, IL-4, IL-1- β , IL-1- α , tumour necrosis factor (TNF- α), and lymphotoxin- α [5, 6]. These cytokines are likely released into the systemic circulation, since animal models indicate that proinflammatory cytokine concentrations are higher within the serum of animals with periapical lesions. The

concentrations of proinflammatory cytokines are also elevated within both the serum and gingival tissues of persons with periodontal inflammation, and may contribute to a systemic hyperinflammatory state, which is a risk factor for several types of systemic diseases [7].

The role of TNF- α in host defence and inflammatory responses is well documented [8, 9]. TNF- α is reported to promote the inflammatory cell infiltration by leukocyte adhesion molecules on endothelial cells and activate phagocyte killing mechanisms. TNF- α is a proinflammatory cytokine that was originally discovered as a protein with necrotizing effects in certain transplantable mouse tumors, and is now recognized as a cytokine with pleiotropic biological capacities. Besides its cytostatic and cytotoxic effects on certain tumour cells, TNF- α influences growth, differentiation, and/or the function of virtually every cell type investigated. Moreover, TNF- α is thought to be part of an integral network of interactive signals that orchestrate inflammatory and immunological events [8, 9].

IL-6 is a multifunctional cytokine playing a central role in inflammation and tissue injury [10]. Its levels positively correlate with higher all-cause mortality, unstable angina, propensity to diabetes and its complications, hypertension, and obesity. Moreover, proinflammatory cytokines were revealed to be sensitive systemic markers of tissue damage, and predictive of future adverse cardiac events among apparently healthy men. IL-6 and TNF- α levels have been shown in periapical lesions and in the liver of rats with induced periapical abscesses [10].

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IL-6 and TNF- α are two cytokines traditionally considered to be key mediators of acute inflammation [9, 11]. Both factors are also crucial for the development of specific immune responses, as mice lacking IL-6 or TNF- α gene expression are deficient in T and B cell function. Inflammation and immune induction can be viewed as intimately linked events as the successful resolution of the inflammatory process leads to the transition from innate to adaptive immunity. Of the two cytokines, IL-6 in particular – through differential control of leukocyte recruitment, activation and apoptosis – has emerged as one of a network of mediators directing this shift from innate to specific immunity [11]. Importantly, IL-6 has also been implicated in the regulation of lymphocyte trafficking through the lymph node following an inflammatory stimulus [11].

Cytokines like TNF- α and IL-1 are strong inducers of interleukin-8 (IL-8). IL-8 is an inflammatory and chemotaxis factor belonging to the CXC family of proteins with glutamate-leucine-arginine (ELR) motif near the N-terminus of the molecule. IL-8 activates multiple intracellular signalling pathways downstream of two cell-surface receptors CXCR-1 and CXCR-2 [12]. IL-8 is synthesized by a vast number of different cells, including monocytes, macrophages, T-cells, neutrophils, fibroblasts, endothelial cells and chondrocytes. The main function of IL-8 is to attract neutrophils and activate them [13, 14, 15, 16]. IL-8 has been shown to play an important role in tumour growth, angiogenesis and metastasis [17].

There are known research works about the concentration of pro-inflammatory cytokines in saliva in oral lichen planus, aphthous stomatitis, periodontitis, squamous cell carcinoma patients, and control groups [18, 19, 20, 21]. There is no data about the concentration of IL-6, IL-8 and TNF- α in saliva in dental caries patients. The aim of this study was to compare the concentration of pro-inflammatory cytokines IL-6, IL-8 and TNF- α in the saliva of dental caries patients with control group, and find a correlation between cytokines.

MATERIALS AND METHODS

Study Population. 274 youths aged 18 suffering from dental caries were recruited and served as an experimental group, 27 of whom were selected to determine levels of cytokines. 10 healthy subjects, also aged 18, served as the control group. Clinical evaluation procedures included: examination of teeth, periodontal, oral mucosal status, evaluation of malocclusion and collection of saliva samples. Body weight and height were measured using an electronic scale (Seca) and Holtain anthropometer. The results were compared with the updated national reference, and Body Mass Index (BMI) was calculated for each individual in a standard way.

The research protocol was approved by the Committee for Ethics and Supervision on Human and Animal Research, Medical University of Białystok, with informed consent from the patients.

Dentistry criteria. Dental examination was performed in a school surgery using a dental mirror, a probe, and a periodontal probe in the artificial light. The numbers of decayed teeth (D), missing teeth due to caries (M) and filled teeth (F) were evaluated in the dentition [22]. The facial

features and occlusal conditions regarding the three planes, median, orbital and horizontal, were estimated by means of orthodontic diagnostics. The depth of gingival pouches and gingival haemorrhage were examined with a periodontal probe in individual sites in the teeth. Additionally, dental plaque and lesions on the mucus membrane of the oral cavity were evaluated.

Salivary Sample Collection. Saliva was collected by a standard method. Samples from the subjects were collected between 09:00-11:00. All subjects abstained from eating and drinking for 2 h. Unstimulated whole saliva was collected for 10 min by the spitting method. Saliva samples were homogenized and clarified by centrifugation at 10,000 g for 15 min at 4°C. The aliquots of clarified supernatants were stored at -70°C for future use in cytokines measurements.

Detection of Salivary Levels of IL-6, IL-8 and TNF- α . Concentration of IL-6, IL-8 and TNF- α was determined in saliva of patients with dental caries and in control group. After collecting all the salivary samples, the studied cytokines were assessed by ELISA.

Determination of IL-6 in Salivary Samples. Saliva samples were determined by using an enzyme-linked immunosorbent assay kit (R&D Systems Inc., Minneapolis, MN, USA). A monoclonal mouse antibody specific for IL-6 was pre-coated onto a microplate. Standards and samples (100 μ L each) were pipetted into the wells in duplicate and any IL-6 present was bound by the immobilized antibody. The microplate was incubated for 2 h at room temperature. After washing away any unbound substances, an enzyme-linked polyclonal antibody-enzyme specific for IL-6 (200 μ L) was added to each well for 2 h at room temperature. Following a wash to remove any unbound antibody enzyme-reagent, a substrate solution (200 μ L) was added to the wells for 20 min; the colour developed in proportion to the amount of IL-6 bound in the initial step. Colour development was stopped by sulphuric acid, and the intensity of the colour measured by measuring the absorbance at 450 nm. The minimum detectable dose (MDD) of IL-6 was 0.7 pg/mL. The concentrations of the samples were calculated from the standard curve and ranged from 0-300 pg/mL. The results were presented in picogram per milliliter (pg/mL). There was no cross-reactivity with other cytokines.

Determination of IL-8 in Salivary Samples. Saliva samples were determined by using an enzyme-linked immunosorbent assay kit (R&D Systems Inc., Minneapolis, MN, USA). A monoclonal mouse antibody specific for IL-8 was pre-coated onto a microplate. Standards and samples (50 μ L each) were pipetted into the wells in duplicate, and any IL-8 present was bound by the immobilized antibody. The samples were diluted and the microplate incubated for 2 h at room temperature. After washing away any unbound substances, an enzyme-linked polyclonal antibody-enzyme specific for IL-8 (100 μ L) was added to each of the wells for 1 h at room temperature. Following a wash to remove any unbound antibody enzyme-reagent, a substrate solution (200 μ L) was added to the wells for 30 min; colour developed in proportion to the amount of IL-8 bound in the initial step. Colour development was stopped by sulphuric acid and the intensity of the colour measured by measuring



the absorbance at 450 nm. The concentration of IL-8 was multiplied by the dilution factor. The minimum detectable dose (MDD) of IL-8 was 3.5 pg/mL. Concentrations of the samples were calculated from the standard curve and ranged from 0-2000 pg/mL. The results were presented in picogram per milliliter (pg/mL). There was no cross-reactivity with other cytokines.

Determination of TNF- α in Salivary Samples.

Concentration of TNF- α was determined by using a commercially available enzyme-linked immunosorbent assay kit (R&D Systems Inc., Minneapolis, MN, USA). A monoclonal mouse antibody specific for TNF- α was precoated onto a microplate. Standards and samples (200 μ L each) were pipetted into the wells in duplicate, and any TNF- α present was bound by the immobilized antibody. The microplate was incubated for 2 h at room temperature. After washing away any unbound substances, an enzyme-linked polyclonal antibody-enzyme specific for TNF- α (200 μ L) was added to each of the wells for 2 h at room temperature. Following a wash to remove any unbound antibody enzyme-reagent, a substrate solution (200 μ L) was added to the wells for 20 min; colour developed in proportion to the amount of TNF- α bound in the initial step. The colour development was stopped by sulphuric acid, and the intensity of the colour was measured by measuring the absorbance at 450 nm. The minimum detectable dose (MDD) of TNF- α was 1.6 pg/mL. The concentrations of the samples were calculated from the standard curve and ranged from 0-500 pg/mL. The results were presented in picogram per milliliter (pg/mL). There was no cross-reactivity with other cytokines.

Statistical Analysis. Results were expressed as mean \pm standard deviation (SD). The Mann Whitney U test was performed to demonstrate the difference of each cytokine level between dental caries patients and the control group in saliva. A statistically significant difference was defined at $p < 0.05$. All data was processed with Statistica 8.0 (StatSoft, USA).

RESULTS

In dentistry, caries and its intensity in the dentition are defined using the caries intensity index of DMF (decay/missing/filled; D+M+F/number of the examined). In the study group, the mean DMF was 11.33, which indicates a high caries rate of the adolescents examined. Gingivitis, according to the gingival index in the Loe evaluation [23], was estimated as marginal gingivitis both in the control and study group. The index was not higher than the clinically significant value 1 (Tab. 1).

The mean BMI and diet between the control and experimental groups were comparable. The mean value of BMI was 22.12 ± 4.59 in control group, and 21.39 ± 9.05 in dental caries patients, This was not statistically significant ($p = 0.387$) (Fig. 1).

The snacks most frequently consumed in both groups (Tab. 2), were: fruit (70.0% vs. 78.1%), yoghurts (50.0% vs. 62.4%), sweets (60.0% vs. 62.8%), sandwiches (70.0% vs. 57.3%), sweet buns, cakes, doughnuts (20.0% vs. 46.0%), fast food meals (10.0% vs. 17.5%), respectively (multiple choice answer).

Table 1. Clinical profile of the study population

	Groups	N	Mean	SD	p
DMF	Control	10	6.7	5.29	0.002**
	Experimental	274	11.33	3.85	
	All	284	11.17	3.99	
GI	Control	10	0.24	0.33	0.733
	Experimental	274	0.3	0.41	
	All	284	0.3	0.41	
PI	Control	10	0.42	0.33	0.919
	Experimental	274	0.53	0.61	
	All	284	0.52	0.6	

** $p < 0.05$ – Differences between patients with dental caries and healthy group. DMF – decay/missing/filled index; PI – plaque index; GI – gingival index.

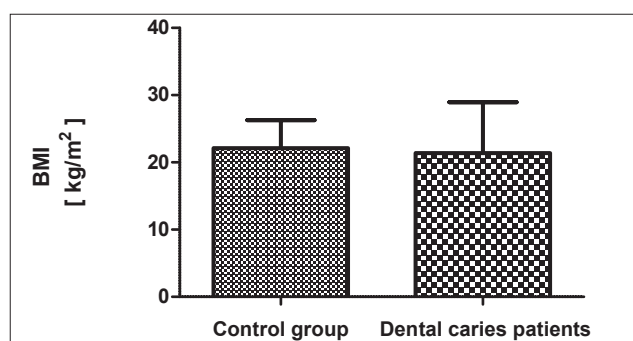


Figure 1. BMI measured in kg/m² in control subjects, compared with levels in dental caries patients

Table 2. Characteristic of diet-snacking

Snacking	Control group N=10		Dental caries patients N=274		p
	n	%	n	%	
Fruit	7	70.0	214	78.1	0.543
Joghurts	5	50.0	171	62.4	0.426
Sweets	6	60.0	172	62.8	0.858
Sandwiches	7	70.0	157	57.3	0.423
Sweet buns, cakes, doughnuts	2	20.0	126	46.0	0.104
Fast food	1	10.0	48	17.5	0.535

Among the preferred beverages were: juice and fruit drinks (40.0% vs. 31.0%), sweetened, non-carbonated drinks (30.0% vs. 31.2%) sweetened carbonated beverages, such as cola type drinks and orangeade (30.0% vs. 28.8%) in the compared groups, respectively. Additionally, 30.0% of young people in the control group and as many as 47.5% of the study group declared that they added extra sugar to their drinks and meals (Tab. 3).

Table 3. Characteristic of beverages

Types of beverages	Control group N=10		Dental caries patients N=274		p
	n	%	n	%	
Fruit juices and fruit drinks	4	40.0	85	31.0	0.546
Sweetened carbonated beverages	3	30.0	87	31.2	0.907
Sweetened fizzy drinks	3	30.0	79	28.8	0.936
Additionally sweetened drinks and meals	3	30.0	130	47.5	0.276

The presented study indicated a high consumption of sweets and sweetened beverages in the study group. As many as 36.5% of the study respondents consumed sweets every day, 38.4% – several times a day, 19.3% – several times a month, while only 5.8% consumed no sweets at all. In the control group, 10.0%, admitted that they ate sweets every day, 70.0% – several times a week, 10.0% – several times a month, whereas 10.0% of respondents declared they had given up eating sweets (Tab. 4).

Table 4. Frequency of consumption (sweets and sweetened beverages)

Consumption of sweets and sweetened beverages	Control group N=10		Dental caries patients N=274		p
	n	%	n	%	
Every day	1	10.0	100	36.5	0.084
Several times a week	7	70.0	105	38.4	0.043**
Several times a month	1	10.0	53	19.3	0.458
No consumption	1	10.0	16	5.8	0.585

**p < 0.05 – Differences between patients with dental caries and healthy group.

In the study group, only 34.3% reported they did not consume fast food, whereas 54.0% ate it several times a month, and 11.7% several times a week. In the control group, 60.0% did not eat this type of food and 40.0% ate fast food several times a month (Tab. 5).

Table 5. Frequency of consumption (fast food)

Consumption of fast food	Control group N=10		Dental caries patients N=274		p
	n	%	n	%	
Several times a week	0	-	32	11.7	0.250
Several times a month	4	40.0	148	54.0	0.381
No consumption	6	60.0	94	34.3	0.094

The levels of IL-6, IL-8 and TNF- α increased statistically in the dental caries group, more so than those in the controls. The highest significance ($p = 0.002$) was observed when TNF- α in saliva was compared to its corresponding control group (Tab. 6). The mean level of salivary IL-6 was 18.5 pg/mL (range: 0.68-107) in dental caries patients and 2.68 pg/mL (range: 0.6-17.3) in the control group (Fig. 2). Statistically, salivary IL-6 levels were significantly higher in dental caries patients ($p=0.005$) (Tab. 6). The mean level of salivary IL-8 was 1,489.24 pg/mL (range:198.18-3,405.21) in dental caries patients, and 619.19 pg/mL (range: 250-1,144) in the control group (Fig. 3). Statistically, salivary IL-8 levels were significantly higher in dental caries patients ($p=0.008$) (Tab. 6). The mean level of salivary TNF- α was 36.5 pg/mL (range: 0.88-216) in dental caries patients, and 7.32 pg/mL (range: 2-23.1) in the control group (Fig. 4). Statistically,

Table 6. Change in IL-6, IL-8 and TNF- α salivary levels in dental caries patients and control

Cytokines	Control group (n = 10)	Dental caries patients (n = 26)	p value
IL-6 (mean \pm SD)	2.68 \pm 5.51	18.50 \pm 27.72	< 0.005**
IL-8 (mean \pm SD)	619.19 \pm 311.79	1489.24 \pm 960.32	<0.008**
TNF- α (mean \pm SD)	7.32 \pm 6.98	36.50 \pm 41.46	<0.002**

**p < 0.05 – Differences between patients with dental caries and healthy group.

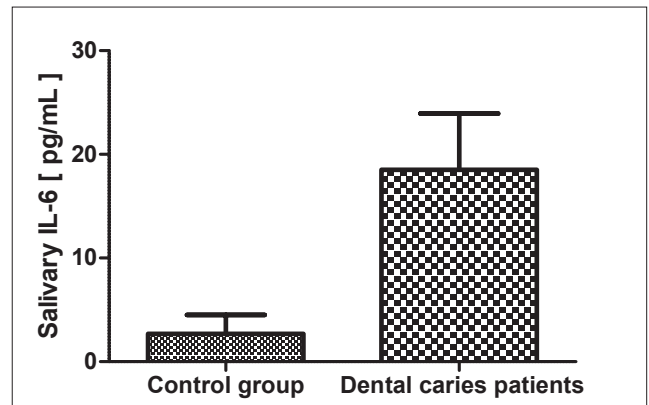


Figure 2. Salivary levels of IL-6 measured in pg/mL in control subjects, compared with levels in dental caries patients

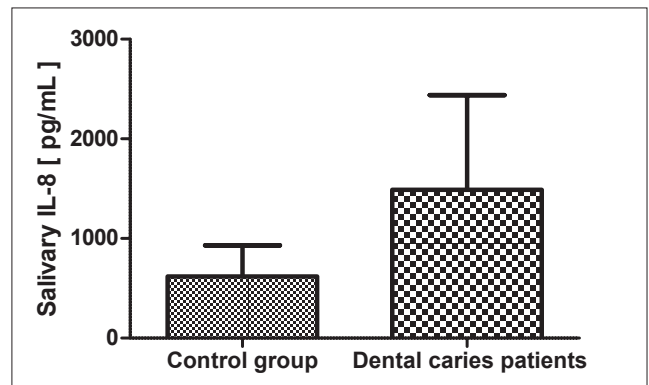


Figure 3. Salivary levels of IL-8 measured in pg/mL in control subjects, compared with levels in dental caries patients

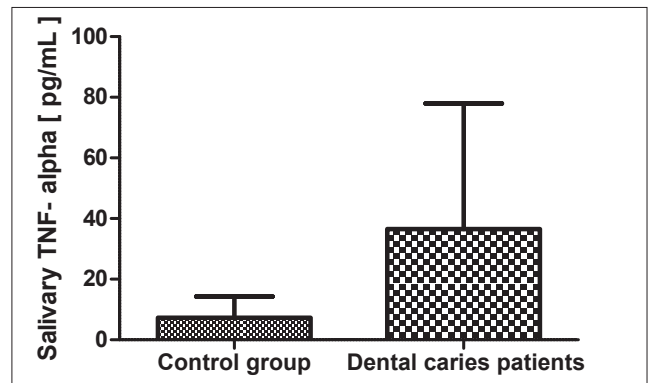


Figure 4. Salivary levels of TNF- α measured in pg/mL in control subjects, compared with levels in dental caries patients

salivary TNF- α levels were significantly higher in dental caries patients ($p=0.002$) (Tab. 6).

The correlation between IL-8 and TNF- α was positive and statistically significant $p=0.009$. There was no significant correlation between cytokines and dentistry parameters (Tab. 7).

Table 7. Correlation between cytokines and correlation between dentistry parameters and cytokines in dental caries patients

Parameters	DMF	GI	PI	IL-6	IL-8	TNF- α
IL-6	0.142	0.156	0.149		0.784	0.295
IL-8	0.285	0.352	0.255	0.784		0.009**
TNF- α	0.675	0.770	0.341	0.295	0.009**	

**p < 0.05 – Differences between patients with dental caries and healthy group.

DISCUSSION

Dental caries is initiated by a bacterial biofilm on the surface of the teeth, below the gingival margin. There are different predisposing factors and aetiologies of this erosive condition. Chemical, biological and behavioural factors are crucial and help explain how some individuals exhibit more erosion than others. Several studies have shown the erosive potential of acidic drinks and foodstuff [25].

A high consumption of high calorie sweets and sweet beverages was revealed, especially, in the study group. Although these products provide a sense of being full, they do provide the organism with an appropriate amount of nutrients. They are high in calories and have a low nutritional value. Simple sugars, found in sweets, are the best substrate for caries-forming bacteria, which by means of enzymatic conversion, produce acids which demineralize dental hard tissue [26]. The frequent consumption of sweetened, non-carbonated and carbonated drinks, including drinks of the Cola type, is of great importance. Coca-cola has potent erosive properties, and due to its acidic content, leads to enamel erosion when consumed frequently and for a long period of time [27, 28].

The damaged dental structure becomes the site prone to the development of caries. The constant increase in the concentration of sugars provided in food results in adaptive changes of bacteria in the dental plaque. Bacteria become more acid-resistant and acid-forming, even several-fold, which constitutes the high caries-forming factor. Until fairly recently it was assumed that the bacteria were the primary cause of tissue destruction; however, a large body of research has revealed that it is the patient's immune response that is actually responsible for the majority of the breakdowns of tooth-supporting tissues.

The purpose of therapy is to restore the balance of pro-inflammatory or destructive mediators and anti-inflammatory or protective mediators to that seen in healthy individuals [2]. More information is needed about the molecular mechanisms connected with dental caries. It is known that pro-inflammatory cytokines play role in such a mechanism, but the details remain unexplained. Cytokines, with other factors, will be useful tools for diagnosis and monitoring the oral cavity, and saliva could be used as a non-invasive diagnostic fluid to measure biomarkers released during disease initiation and progression. Cytokines regulate many aspects of the immune response. In leukocyte trafficking, cytokines have been shown to influence the expression of adhesion markers and, quite possibly, associated chemokines. In the innate response, oral microbial pathogen-associated molecular patterns bind to pattern-recognition receptors on host cells, including dendritic cells, which then activate the inflammatory response with a release of pro-inflammatory cytokines, such as IL-1 β , IL-6, IL-8, and TNF- α . Initially, only gingivitis might develop, and if the immune response is successful, the gingivitis will resolve; however, if the infection persists and further proliferation of the bacteria occurs, intensification of the inflammatory response can lead to the destruction of periodontal tissue in susceptible subjects.

The presented study was undertaken to test the hypothesis that the elevation of salivary pro-inflammatory cytokines – IL-6, TNF- α and chemokine – IL-8, changed in dental caries patients. TNF- α is known to be produced predominantly by activated macrophages at the site of infection/inflammation,

and also by T cells. IL-6, on the other hand, can be generated not only by activated macrophages/monocytes, but also by fibroblasts and activated endothelial cells in inflamed tissue. In turn, IL-6 production can lead to the inhibition of TNF- α expression [11].

The obtained results show that the elevation of IL-6, IL-8 and TNF- α in unstimulated whole saliva in subjects with dental caries, compared with controls, increased and was statistically significant in all cases ($p < 0.05$). It is well known that the salivary level of cytokines may be increased as a result of disorders in the oral cavity. Researchers have speculated that dysfunction of the triangle oral mucosa, immune cells and saliva leads to the onset of oral mucosa diseases and inflammation, such as oral lichen planus, oral cancer, periodontitis and leukoplakia [28].

TNF- α , IL-6 and IL-8 products of immune cells also play an important role in diseases of the oral mucosa, but the exact role played by cytokines in the aetiology of dental caries is not well known. The higher level of TNF- α was measured in patients with some inflammatory diseases: oral lichen planus (OLP), aphthous stomatitis (RAS), oral squamous cell carcinoma (OSCC). The results were statistically significant in OLP and RAS patients, but was not statistically significant in OSCC patients [18, 19, 20, 21]. Researchers have found higher concentration of IL-6 in OSCC [19]. The levels of inflammatory cytokine (IL-6, 8 and TNF- α) were determined in saliva in chronic periodontitis and periodontally healthy subjects. The level of IL-6 and TNF- α was higher, but IL-8 lower, in a periodontally healthy group in comparison with chronic periodontitis [29].

There are also some research studies that show lower concentrations of pro-inflammatory cytokines after different therapies. Ghallab showed a lower elevation of TNF- α in erosive OLP patients after treatment with prednisone [21].

Higher levels of TNF- α and IL-6 in dental caries patients lead to a lower number of osteoblasts and fibroblasts, and they support the demineralization of teeth and development of the dental caries process [30]. IL-6, with other factors, causes the resorption of bones and stimulates the synthesis of chemokines [31]. Tani-Ischii et al. proved that TNF- α , which is locally produced by osteoclasts, is a very important factor regulating differentiation of these cells and takes part in the processes of resorption [32].

The presented study shows the positive correlation between TNF- α and IL-8. TNF- α is an effector cytokine and its production leads to oral diseases [28]. It is probable that the higher level of IL-8 induces TNF- α . These two cytokines play an important role in immunity of the oral cavity. Taken together, these data suggest links between the production of inflammatory and immunoregulatory cytokines and chemokines, such as the tumour necrosis factor α (TNF- α), interleukin-6 (IL-6), interleukin-8 (IL-8), and dental caries disease.

CONCLUSIONS

The presented data are based on very small group and the findings require further investigation. The results suggest links between the production of tumour necrosis factor α (TNF- α), interleukin-6 (IL-6), interleukin-8 (IL-8) in saliva and dental caries disease. Tumour necrosis factor α (TNF- α), interleukin-6 (IL-6) and interleukin-8 (IL-8) secretion in

saliva will be useful tools for diagnosing and monitoring dental caries disease, and saliva could be used as a non-invasive diagnostic fluid to measure biomarkers released during disease initiation and progression.

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