The results of dysbiosis in the structure of periodontopathogens of the II order in patients with non-alcoholic fatty liver disease

Objective — to identify the quantitative composition of some representatives of II order periodontopathogens in patients with NAFLD.

Materials and methods. 108 people were selected, including 44 patients with non-alcoholic fatty liver disease (main group), 44 people — their spouses (comparison group). The control group consisted of 20 somatically healthy volunteers. A standard dental examination was performed using hygiene and periodontal indices, and some physical parameters of saliva (salivary salinity and viscosity, oral fluid endotoxin level in volume per unit time by enzyme-linked immunosorbent assay) were determined. Quantitative composition of Fusobacterium nucleatum, Treponema denticola, Prevotella intermedia and Porphyromonas endodontalis in periodontal pockets was performed by real-time PCR using paper endodontic absorbents. Statistical processing was performed using SPSS statistical programs using the nonparametric method.

Results. In NAFLD, negative changes in the physical parameters of saliva were observed (decreased salivary flow rate, increased viscosity, pH shift to the acidic side), increased levels of oral endotoxin and increased quantitative content of periodontal pathogens of the second order (increase in Porphyromonas endodontalis compared to the control group by more than 4.5 times and increase in Prevotella intermedia by 2.5 times). A positive correlation was found between the presence of halitosis and the quantitative content of Fusobacterium nucleatum \( (r = 0.40; p = 0.065) \) and the quantitative content of Porphyromonas endodontalis \( (r = 0.36; p = 0.022) \). We also found a negative correlation between the level of Fusobacterium nucleatum and salivary flow rate \( (r = -0.51; p = 0.000) \) and a positive correlation with saliva viscosity \( (r = 0.49; p = 0.001) \).

Conclusions. The combination of exogenous and endogenous risk factors forms a dysbiotic shift of conditionally pathogenic microflora (periodontal pathogens of the second order) in the oral cavity biotope, which creates conditions for the invasion of more aggressive microorganisms and the maintenance of chronic systemic inflammation. This fact requires increased attention from both the dentist and the internist, and the patient.

Keywords: chronic periodontitis, endotoxin, oral microbiome.
microbiome) [13]. Increased number of periodontopathogens contributes to changes in the phenotype due to the expression of genes with boosted virulence and pathogenicity in relation to periodontal tissues. According to the Socransky theory, periodontopathogens form certain microbial complexes that differ in phylotypes and pathogenicity [21].

Most often, three types of bacteria are found in the CP foci i.e., Porphyromonas gingivalis, Aggregatibacter and Tannera forsythia. In terms of virulence and pathogenicity to periodontal tissues, they are the most aggressive [22]. In addition, they can modulate immune system responses in their favor, thereby allowing them self-protecting against antimicrobial activity. The above microorganisms belong to the I order periodontopathogens due to the diversity and aggressiveness of their own virulence factors. However, I order periodontal pathogens are microorganisms of late colonization in the structure of a pathogenic biofilm and therefore require the formation of certain environmental conditions in an eoniche for their own infectivity.

The presence of concomitant somatic pathology in a dental patient, in particular, non-alcoholic fatty liver disease (NAFLD), contributes to a decrease in the adaptive capabilities of the periodontium to constant action of various exogenous risk factors and the formation of conditions in the biotope i.e., the oral cavity with dysbiotic shift of pathogenic microflora and high virulence [18, 20]. An increased level of endotoxemia, hypoxia and energy deficiency backed by NAFLD contribute to the weakening of the gingival epithelium barrier function and invasion of periodontopathogens into the periodontium.

Amongst periodontal pathogens, there is also a II order group, which includes various representatives of resident microflora, but inferior to those from the I order group by a moderate set of virulence factors and less aggression towards periodontal tissues [1, 11]. However, according to the literature, their main function in the presence of a dysbiotic shift in the microbiome structure is the formation of favorable environmental conditions in the biotope i.e., the oral cavity for the growth and progression of the I order periodontopathogens. In addition, the II order periodontopathogens play the role of an anchor for the co-adhesion of pathogenic microflora of later colonization in the biofilm structure and, by combining with them into complexes, complement the virulence factors.

A quantitative increase in the number of the II order periodontopathogens in the econiche-gingival sulcus in a patient with NAFLD can be considered a marker for the formation of favorable conditions for the growth of more aggressive microorganisms with subsequent progression of CP.

Objective — to identify the quantitative composition of some representatives of II order periodontopathogens in patients with NAFLD.

Materials and methods

Ethical part

The study was conducted in accordance with the Declaration of Helsinki. All patients enrolled in the study signed an informed consent form, which stipulated the possibility of early withdrawal from the study if desired.

Clinical part

The study involved 44 patients with a verified NAFLD diagnosis (main group), their spouses (comparison group), and 20 somatically healthy volunteers from the control group. The total number of subjects was 108. The median and interquartile range of NAFLD patients age were 50.0 [42.0; 58.0] years, their family members — 47.0 [39.0; 53.0] years. Control group patients were representative in terms of gender and age.

NAFLD diagnosis was established according to national and worldwide criteria (except for other possible factors facilitating the development of secondary hepatic steatosis) [6, 17]. To diagnose and classify the degree of obesity, WHO criteria (estimated body mass index (BMI)) were used. All subjects were asked about daily oral hygiene and dietary habits.

Dental examination was carried out according to standard methods with the determination of hygienic index OHI-S and periodontal indices. Periodontal pathological changes diagnosis was verified according to the classification of diseases and conditions of periodontal and peri-implant tissues (EFP & AAP World Workshop, 2018) [10]. In order to obtain data homogeneity, patients were warned not to smoke, chew gum, or perform oral hygiene procedures before manipulation.

Lab part

The state of the secretory function was determined in terms of the rate of salivation of mixed saliva, evaluating its received volume per unit of time. Unstimulated oral fluid was collected without stimulation on an empty stomach in graduated tubes for 10 minutes. Also, the viscosity of the oral fluid, its pH were determined using test strips.

Saliva bacterial endotoxin concentration was determined by enzyme immunoassay using the LAL Chromogenic Endpoint Assay kit (Hycult Biotech, the Netherlands). The range for determining the concentration is from 0.01 to 10 U/mL. Sensitivity — 0.01 U/mL.

Quantitative composition of Fusobacterium nucleatum, Treponema denticola, Prevotella intermedia
and *Porphyromonas endodontalis* was determined by real-time quantitative polymerase chain reaction (qRT-PCR) using universal primers. Material was taken from periodontal pockets using paper endodontic absorbers (AbsorbentPaperPoints, by Maillefer (size No. 25). The absorbers were left in the pockets for 15—20 seconds with minimal contact with atmospheric air, and thereafter were immediately transferred to an Eppendorf-type tube with DNA-EXPRESS reagent, which is used for rapid lysis of biomass, and forwarded to the laboratory. 4 absorbers were used for each participant.

**Statistical part**

Data obtained was statistically processed using the SPSS statistical software package (SPSS). To test the sign for normality, the Kolmogorov-Smirnov test was used. Given the distribution of studied characteristics, nonparametric methods were used to describe and compare the indicators, the distribution of which differed from the normal one: calculation of the median and interquartile range Me [25; 75]; Mann-Whitney criterion. A comparison of qualitative features, as well as a study of the frequency of detection of indicators, was carried out using criterion 2 and the analysis of contingency tables. Correlations were evaluated by the Pearson correlation ratio for quantitative traits distributed according to the normal law, by the Spearman ratio for other types of distribution, and by the Kendal ratio for qualitative and quantitative indicators.

**Results**

First of all, the presence of changes in such physical properties of saliva as salivation rate, viscosity and pH was revealed. The results obtained differed as expected when comparing groups with each other. Thus, it should be noted that in the main group patients, the rate of salivation had the lowest values, while the viscosity of oral fluid significantly exceeded the norm, and the pH was below the lower limit of the norm by more than 0.3. These results are presented in Table 1.

The analysis of quantitative fluctuations of some representatives of the II order periodontopathogens in the studied groups showed significant differences in the results among such bacteria as *Fusobacterium nucleatum*, *Treponema denticola*, *Prevotella intermedia* and *Porphyromonas endodontalis* (Table 2). It should be noted that these bacteria are directly involved in the formation of comfortable environmental conditions in the biotope for further infectivity and invasion by the I order periodontal pathogens, due to the coadhesion with the latter and an increase in their virulence factors in relation to periodontal tissues.

### Table 1. Comparison of physical indicators of mixed saliva in groups

<table>
<thead>
<tr>
<th>Index</th>
<th>Control group</th>
<th>Comparison group</th>
<th>Main group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salivation rate, mL/min</td>
<td>0.50 [0.40; 0.55]</td>
<td>0.40 [0.31; 0.50]*</td>
<td>0.25 [0.20; 0.30]**</td>
</tr>
<tr>
<td>Viscosity, rel.</td>
<td>2.40 [2.10; 2.60]</td>
<td>2.80 [2.02; 3.07]</td>
<td>3.2 [2.67; 3.80]**</td>
</tr>
<tr>
<td>pH</td>
<td>6.90 [6.70; 7.00]</td>
<td>6.80 [6.62; 6.90]</td>
<td>6.5 [6.30; 6.60]**</td>
</tr>
</tbody>
</table>

Note. The difference from the control group is statistically significant: *p < 0.01; **p < 0.001.
The difference from the comparison group is statistically significant: *p < 0.001.

### Table 2. Quantitative level of the II order periodontopathogens in groups

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Control group</th>
<th>Comparison group</th>
<th>Main group</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusobacterium nucleatum</em>, Log/ml</td>
<td>4.51 [3.57; 5.33]</td>
<td>4.62 [3.68; 5.18]</td>
<td>5.2 [4.60; 5.80]**</td>
</tr>
<tr>
<td><em>Treponema denticola</em>, Log/ml</td>
<td>2.5 [0.28; 4.05]</td>
<td>2.79 [0.60; 4.10]</td>
<td>2.58 [0.00; 4.16]</td>
</tr>
<tr>
<td><em>Prevotella intermedia</em>, Log/ml</td>
<td>0.63 [0.00; 1.46]</td>
<td>0.89 [0.00; 1.83]</td>
<td>1.69 [0.00; 4.25]**</td>
</tr>
<tr>
<td><em>Porphyromonas endodontalis</em>, Log/ml</td>
<td>0.0 [0.00; 3.32]</td>
<td>0.55 [0.00; 4.53]</td>
<td>4.47 [3.16; 5.59]***</td>
</tr>
</tbody>
</table>

Note. The difference from the control group is statistically significant: *p < 0.05.
The difference from the comparison group is statistically significant: *p < 0.05; **p < 0.01; ***p < 0.001.
The infectivity of aforesaid II order periodontopathogens also contributed to the increased level of endotoxin (LPS) in saliva. As it is known, LPS is a structural element of the outer membrane of a bacterial cell, and also has antigenic properties and has a genotoxic effect on the gingival epithelium, weakening its barrier capabilities. Data on the level of LPS in saliva are presented in Table 3.

It is important to note that periodontal pathogens are anaerobes, and most of them are obligate, therefore the complaint of patients about bad breath (halitosis) attracted special attention. Thus, the main group patients (86.4%) suffered from halitosis most often, while their spouses mentioned this fact only in 27.3% of cases. Also, based on the history data, it is possible to talk about the presence of certain bad habits, namely smoking, excessive abuse of sweets, namely: more than 80.0 % of those with NAFLD and 54.5 % of their family members preferred carbohydrate foods and/or sugary carbonated drinks, and almost half of the respondents from these groups smoked. It is also worth noting that in the control group, not a single patient noted bad breath, and a third of patients noted excessive consumption of carbohydrates and sweets.

According to the survey, only control group subjects (100.0 %) adhered to correct double oral hygiene algorithm, while the majority of patients in the main and comparison groups brushed their teeth only once a day and not always regularly, which was confirmed by unsatisfactory state of hygiene according to the Green-Vermilion (OHI-S) hygiene index (Figure).

The analysis of possible dependence of obtained indicators made it possible to identify both positive and negative correlations. Thus, a positive correlation was found between the presence of halitosis and *Fusobacterium nucleatum* (*r* = 0.40; *p* = 0.065) and *Porphyromonas endodontalis* (*r* = 0.36; *p* = 0.022). Sweets abuse has shown an unexpected relationship with the II order periodontopathogens. Thus, excessive consumption of sugary carbonated drinks was positively correlated with *Fusobacterium nucleatum*, and the same indicator had a negative correlation with *Prevotella intermedia* (*r* = −0.35; *p* = 0.020).

When analyzing correlations with saliva physical parameters, reliable results were obtained only with *Fusobacterium nucleatum* i.e., a negative correlation with the rate of salivation (*r* = −0.51; *p* = 0.000) and a positive correlation with the oral fluid viscosity (*r* = 0.49; *p* = 0.001).

**Discussion**

The importance of protecting the structure of healthy oral microbiome is undeniable. The mechanisms of colonization resistance provided by the resident microflora allow maintaining correct balance between gram-positive and gram-negative bacteria in the oral cavity biotope, controlling the required amount of opportunistic microflora and, in general, contributing to correct interaction between local microbiome [4]. The oral cavity biotope is characterized by comfortable environmental conditions and forms several econiches that differ in the composition of resident microflora. An important econiche, as a barrier between oral cavity and internal body environment, is the periodontium, namely the gingival sulcus [7]. In addition, one of the econiches is also saliva/oral fluid, which covers the mucous membrane and provides certain comfortable conditions for the life of the resident microflora, namely, humidity, pH constancy, and the source of trophic substrates. Also, constant and controlled salivation contributes to the function of cleaning from soft plaque, desquamated epithelium, bacteria, etc. from mucosal surface [15].

<table>
<thead>
<tr>
<th>Index</th>
<th>Control group</th>
<th>Comparison group</th>
<th>Main group</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS, U/mL</td>
<td>20.6 [11.7; 23.4]</td>
<td>26.75 [19.4; 39.5]</td>
<td>33.5 [23.8; 52.3]*</td>
</tr>
</tbody>
</table>

Note. The difference from the control group is statistically significant: *p* < 0.01.

**Table 3. LPS level of oral fluid in study groups**

---

**Figure. Oral hygiene quality according to OHI-S index**

Note. * The difference is significant when compared with the control group.

*The difference is significant when compared with the comparison group.
However, in NAFLD, basic physical properties of saliva do change, and it turns from a protection factor into a source of dysbiotic shift.

According to Table 1, in NAFLD, the viscosity of oral fluid increases, while the rate of salivation decreases, which characterizes a violation of the cleaning function performed by saliva in relation to various oral cavity tissues. According to the most researchers, the viscosity of saliva is provided by the presence of proteins in its composition (mucins, glycoproteins, fibronectins etc.), but their quantity in a healthy state of the body is normalized only for a positive effect [3, 8]. Proteins are necessary for surface tension and retention of the layer of saliva on the mucous membrane surface; to protect the epithelium of the gums, they are used by some representatives of resident microflora as a trophic substrate, and their metabolic products, as energy substances, by other microorganisms. However, keeping in mind that saliva/oral fluid is considered one of the econiches of the oral cavity biotope, it becomes clear that increased level of proteins in its composition allows them to be used as a scaffold for adhesion and growth of opportunistic microflora microcolonies [14]. Therefore, increased viscosity and decreased rate of salivation will contribute to the transformation of saliva/oral fluid into an econiche for the growth of the II order periodontopathogens. Decreased saliva pH in the main group patients may also indicate the quantitative growth and metabolic activity of these microorganisms, because they are anaerobes by the type of respiration, and also as a result of oxidative stress organisms, because they are anaerobes by the type of respiration, and therefore their metabolic processes contribute to an increase in the concentration of gaseous substances in the exhaled air. Thus, the presence of a halitosis complaint can also be considered a marker of the formation of conditions and the growth of periodontopathogens. Also, the abuse of sweets allows periodontal pathogens to receive an energy substrate for the growth of their own microcolonies and contribute to oxidative stress in periodontal tissues. Of course, such a bad habit as smoking directly contributes to the formation of hypoxia in periodontal tissues, and together with NAFLD is a combination of risk factors for initiating a dysbiotic shift.

Table 2 shows that quantitative level of Treponema denticola is higher in the comparison group (spouses from the main group), where there is no endogenous risk factor of NAFLD. However, in this group there are other exogenous risk factors, such as abuse of sweets in more than half of the studied individuals (54.5%) and smoking in almost every second (47.7%). Treponem denticola is mobile in the oral fluid increased viscosity (see Table 1), unlike the other indicated bacteria, and can independently move in the biotope environment to the econiche-gingival sulcus [12]. It has a sufficiently high adhesion to oral fluid proteins and gum epithelioocytes, which helps to reduce the barrier properties of the mucosa. In addition, this bacterium forms pathogenic complexes of Porphyromonas gingivalis and Fusobacterium nucleatum [16]. Thus, the presence of bad habits that each person can correct/eliminate is already a risk of initiating dysbiotic fluctuations in the oral microbiome structure, which requires attention from both the doctor and the patient himself.

**Conclusions**

Opportunistic microflora is a representative of a healthy oral microbiome; however, its amount is strictly controlled by its own mechanisms of microbiome colonization resistance and therefore its role is absolutely positive. When exogenous risk factors (bad habits) are combined with endogenous ones (NAFLD), conditions are formed in the oral cavity biome marker of the formation of conditions and the growth of periodontopathogens.
II order periodontopathogens are directly involved in the maintenance and development of conditions in the biotope environment for subsequent effectiveness and invasion of more aggressive I order periodontal pathogens. Elimination of bad habits, both with and without NAFLD, related to the eliminated risk factors, and the observance of regular home oral hygiene is the patient's own, motivated contribution and the key to success in treatment and prevention of comorbid diseases.

Conflicts of interest: none.

This study didn’t supported by any source of funding and didn’t included financial interest.

Authorship contributions: conception and design, acquisition, analysis and interpretation of data — D. V. E., N. Y. E.; drafting and critical revision of the article — D. V. E.

References


Д. В. Ємельянов, Н. Ю. Ємельянова
ДУ «Національний інститут терапії імені Л. Т. Малої НАМН України», Харків

Наслідки дисбіозу в структурі пародонтопатогенів ІІ порядку у хворих на неалкогольну жирову хворобу печінки

Мета — визначення кількісного складу деяких представників пародонтопатогенів ІІ порядку у хворих на неалкогольну жирову хворобу печінки (НАЖХП).

Матеріали та методи. Відібрано 108 осіб, з яких 44 пацієнти з неалкогольною жировою хворобою печінки (основна група), 44 особи — їхні подружжі (група порівняння). Контрольну групу склали 20 соматично здорових добровольців. Проведено стандартне стоматологічне обстеження з використанням індексів гігієни та пародонта, визначено деякі фізичні параметри слизни (рівень салівації та в'язкість слизни, рівень ендотоксину ротової рідини в об'ємі за одиницю часу імунокомунісним методом). Кількісний склад Fusobacterium nucleatum, Treponema denticola, Prevotella intermedia і Porphyromonas endodontalis у пародонтальних кишенях проводили за допомогою ПЛР-дослідження в умовах реального часу з використанням паперових ендодонтичних абсорбентів. Статистична обробка здійснювалася за допомогою статистичних програм SPSS непараметричним методом.

Результати. При НАЖХП спостерігалися негативні зміни фізичних параметрів слизни (зниження швидкості слиновиділення, підвищення в'язкості, зсув рН у кислий бік), підвищення рівня ендотоксину ротової рідини і збільшення кількісного вмісту пародонтопатогенів ІІ порядку (збільшення Porphyromonas endodontalis у порівнянні з групою контролю більше ніж у 4,5 разу та збільшення рівня Prevotella intermedia в 2,5 разу). Виявлено позитивний зв'язок між наявністю галітозу та кількісним вмістом Fusobacterium nucleatum (r = 0,40; p = 0,065) і кількісним вмістом Porphyromonas endodontalis (r = 0,36; p = 0,022). Також встановлено позитивну залежність рівня Fusobacterium nucleatum від швидкості слиновиділення (r = – 0,51; p = 0,000) та позитивну залежність від в'язкості слизни (r = 0,49; p = 0,001).

Висновки. Поєднання екзогенних та ендогенних факторів ризику формує дисбіотичний зсув умовно-патогенної мікрофлори (пародонтопатогенів ІІ порядку) у біотопі — порожнині рота, що створює умови для інвазії більш агресивних мікроорганізмів та підтримання хронічного системного запалення. Цей факт вимагає підвищеної уваги як з боку лікаря-стоматолога та лікаря-інтерна, так і самого пацієнта.

Ключові слова: хронічний пародонтит, ендотоксин, мікробіом порожнини рота.

ДЛЯ ЦИТУВАННЯ
