

# ОРИГІНАЛЬНІ ДОСЛІДЖЕННЯ

DOI: 10.21802/acm.2022.1.8

UDC 612.015.3+546.17+546.221.1+612.313+616.314-77

## CHANGES IN THE METABOLISM OF NITRIC OXIDE, HYDROGEN SULFIDE OF THE ORAL FLUID TAKING INTO ACCOUNT AGE AND DENTAL STATUS

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**Abstract.** The relevance of the study is conditioned by the prevalence of dental pathology, its complicated course in senior age and the search for the age-associated metabolic markers of oral fluid.

**Objective.** The aim of the study is: to determine the age peculiarities of the content of nitric oxide and hydrogen sulfide metabolism in the oral fluid of patients, taking into account their dental status.

**Materials and Methods.** Indicators of nitric oxide metabolism, L-arginine/arginase, hydrogen sulfide content in oral fluid, dental status of different aged people under conditions of intact dentitions, their defects and against the background of correction with different types of dentures, were studied.

**Results.** It was determined that with age, changes in oral fluid are characterized by an increase in nitrite ion, the amount of nitrites and nitrates, increasing the formation of peroxynitrite against the background of lower levels of L-arginine and hydrogen sulfide, inhibition of arginase activity. The results obtained are confirmed by the presence of close correlations between the concentrations of hydrogen sulfide and peroxynitrite (reverse, strong), caries intensity and: the total level of nitrites and nitrates (direct, strong), peroxynitrite content (direct, medium strength).

**Conclusion.** The studied biomarkers of oral fluid depend more on age, hygienic condition of the oral cavity, intensity of dental caries and defects of dentitions than types of dentures. Therefore, age-related changes in the biochemical composition of oral fluid, metabolic, somatic, comorbid pathologies are likely to be a trigger for structural-functional disorders of the dental-maxillofacial system.

**Key words:** *nitric oxide, nitrites and nitrates, L-arginine, arginase, hydrogen sulfide, oral fluid, dental status*

**Резюме.** Актуальність дослідження зумовлена поширеністю стоматологічної патології, її ускладненим перебігом у старшому віці та пошуком вік-асоційованих метаболічних маркерів ротової рідини.

**Мета дослідження:** з'ясувати вікові особливості вмісту показників метаболізму оксиду азоту та сірководню у ротовій рідині пацієнтів з урахуванням стоматологічного статусу.

**Матеріали і методи.** Досліджували показники системи метаболізму оксиду азоту, L-аргінін/аргінази, вміст сірководню у ротовій рідині, стоматологічний статус людей різного віку за умов інтактних зубних рядів, їх дефектів та на тлі корекції різними видами зубних протезів.

**Результати.** Установлено, що з віком зміни ротової рідини характеризуються підвищенням вмісту нітрит-іона, суми нітритів і нітратів, зростанням утворення пероксинітриду на тлі зниження рівня L-аргініну та сірководню, пригнічення активності аргінази. Отримані результати підтверджені наявністю тісних кореляційних взаємозв'язків між концентраціями сірководню та пероксинітриду (зворотний, сильний), інтенсивністю карієсу та: сумарним рівнем нітритів і нітратів (прямий, сильний), вмістом пероксинітриду (прямий, середньої сили).

**Висновки.** Досліджувані біомаркери ротової рідини в більшій мірі залежать від віку, гігієни ротової порожнини, інтенсивності карієсу, дефектів зубних рядів, аніж видів зубних протезів. Тому вікові зміни біохімічного складу ротової рідини, метаболічні, соматичні, коморбідні патології з великою ймовірністю можуть виступати тригером структурно-функціональних порушень зубощелепної системи.

**Ключові слова:** *оксид азоту, нітрити та нітрати, L-аргінін, аргіназа, сірководень, ротова рідина, стоматологічний статус*

**Problem statement and analysis of recent research.** Oral fluid is one of the most important factors that maintain the homeostasis of the oral cavity, because it provides trophism of the dentomaxillofacial system. Changes in the oral fluid composition may reflect disorders that occur not only in the structures of the maxillofacial area, but throughout the body [1]. Taking into account the steady trend towards demographic

aging, the study of the peculiarities of indices of metabolism and physicochemical composition of the oral fluid depending on age and dental status attracts attention [2]. The first signs of aging appear at the age of 30-35 years and then gradually increase, reaching a maximum in people over 75 years. The rate and intensity of changes in the organism during aging depend on internal and external factors, in particular, genetic,

bad habits, occupational hazards, habitat and adverse environmental factors. With aging, certain conditions are created for the development of various somatic diseases that can form a combined pathology, reducing the physiological and adaptive capabilities of the body. This also includes dental diseases, especially in people of older age. Due to the decrease in the body's resistance, immune protection with age, one can assume a high probability of complicated pathology in elderly patients. Despite the significant achievements of modern dentistry in the diagnosis of the oral cavity diseases, dental morbidity and the frequency of complications in the treatment of the older age groups is constantly increasing [3, 4]. One of the ways to prevent their development is to increase the efficiency and expand the list of diagnostic methods at the stage of preclinical changes. Therefore, it is important to identify biomarkers of aging, which in the absence of disease can signal of a decrease in the functional capacity and reserve abilities of the body at a certain age.

One of the most common non-invasive methods in dentistry is the biochemical study of oral fluid, which is actively involved in the processes occurring in the oral cavity and plays a significant role in maintaining its homeostasis [1, 5, 6]. Oral fluid biomarkers make it possible to objectively assess the indices of physiological and pathological processes, the informativeness of which characterizes the presence of a blood-salivatory barrier. Their study allows to reflect the physiological state of the structures of maxillofacial area and predict the development of possible diseases [7]. Therefore, the search for new indices to assess the status of local protection in the oral cavity remains an urgent problem.

Nitrogen monoxide (NO) and hydrogen sulfide ( $H_2S$ ) are the indices that have become increasingly relevant in recent years due to their high reactivity and important role in physiological processes. These gas transmitters are actively involved in the regulation of body functions. In particular, NO plays an important role in maintaining vascular tone, affects the immune status and neuronal function [8, 9]. It is well known that in the mechanisms of NO synthesis the role of limiting factor is played by arginase [8]. It is important that NO can have both protective and damaging effects on the mucous membrane of the oral cavity (MMOC) depending on its concentration. It is involved into the synthesis of glycoproteins that have the ability to cover the surface of the MMOC and other parts of the digestive tract, protecting them from harmful factors of physical, chemical and biological origin. NO has a regulatory effect on gastric secretion, motility of the gastrointestinal tract [9]. Significant is the vasodilating effect of NO, which affects the vessels of the MMOC, in particular, the microcirculatory system. This property of NO is important in inflammatory processes, as the

improvement of microcirculation increases tissue trophism. The increase in NO may also be the result of an imbalance between the factors of aggression, in particular, oral infection, and the body's protective reactions.  $H_2S$  stabilizes mitochondrial function, has a pronounced neuroprotective effect in case of tissue damage (in traumas, hypoxia, ischemia), prevents the development of glutamate-induced oxidative stress [10].

**Objective.** The aim of the study is: to determine the age peculiarities of the content of nitric oxide metabolism and hydrogen sulfide in the oral fluid of patients, taking into account their dental status.

### Material and methods

To achieve this goal, there were examined 103 patients without severe concomitant somatic pathology, and they were divided according to age into the following groups: group I – adolescence (18-24 years,  $n = 25$ ), group II – young age (25-44 years,  $n = 21$ ), group III – middle adulthood (45-59 years,  $n = 22$ ), group IV – advanced age (60-74 years,  $n = 19$ ) and group V – senile age (75-89 years,  $n = 16$ ). The examination was performed using a standard set of tools in an inpatient dental office before orthopedic treatment. Defects of dentitions were classified according to Kennedy classification. When examining dentitions, their integrity was taken into account, and the intensity of dental caries was assessed using the DMF index, where D – is decayed (caries), M – is missing (removed), F – is filled (filling). Interpretation of the study results was performed in accordance with WHO recommendations [2]. The condition of periodontal soft tissues was assessed by the values of the papillary-marginal-alveolar index (PMA) in the Parma modification [11]. Hygienic status was determined on the basis of the simplified oral hygiene index according to the Green-Vermillion Oral Hygiene Index Simplified (OHI-S) [10]. The condition of the oral cavity mucous membrane (MMOC) in the examined patients with edentulous jaws was characterized according to the classifications of Schroeder for the upper jaw and Keller for the lower jaw.

Oral fluid was taken in the morning, on an empty stomach, without stimulation, pre-cleaning and rinsing of the oral cavity to prevent qualitative and quantitative changes in the studied parameters. The content of nitrite-ion, the sum of nitrites ( $NO_2^-$ ) and nitrates ( $NO_3^-$ ), L-arginine, peroxy-nitrite, hydrogen sulfide ( $H_2S$ ) and arginase activity were determined in the oral fluid. The determination of  $NO_2^-$  and  $NO_3^-$  content was performed using the reaction with sulfanilamide and N (1-naphthyl)-ethylenediamine in an acidic medium with the use of a mixture of zinc dust and manganese sulfate (to reduce nitrate to nitrite) or manganese sulfate (to determine nitrite-ion) [12]. Determination of L-arginine content was performed using the Sakaguchi reaction [12].

Arginase activity was assessed according to changes in urea concentration [12].  $H_2S$  level was determined according to the reaction with p-phenylenediamine [13].

The examinations were performed with the informed consent of patients for clinical trials, which was approved by the Commission on Bioethics Issues in Ivano-Frankivsk National Medical University. The research was performed in compliance with the "Ethical Principles for Medical Research Involving Human Subjects", approved by the Declaration of Helsinki (1964-2013) and in accordance with ethical and moral-legal requirements of the Ministry of Health of Ukraine №281 dated 01.11.2000.

Statistical processing of the results was performed based on the Exel package of Microsoft Office 365 ProPlus using the methods of variation statistics with the help of Student's t-test. The difference between the studied indices was considered reliable at a value of  $p < 0.05$ . To determine the strength and direction of the relationship between the indices there was used correlation analysis with the use of the Pearson correlation coefficient  $r$ .

### Results and discussion

As a result of the study it was found that the DMF index in the studied patients in group I corresponded to a low level ( $5.20 \pm 0.18$ ), in group II – had a tendency to increase ( $9.70 \pm 0.36$ ), in group III – reached a level of ( $14.15 \pm 1.11$ ), in groups IV and V – was high and amounted to ( $22.80 \pm 2.46$ ) and ( $26.30 \pm 3.50$ ), respectively. According to the classification of dentitions defects, according to the Kennedy method, in patients of groups I and II the 4<sup>th</sup> class dominated, in patients of group III – the 2<sup>nd</sup> class, in groups IV and V – the 1<sup>st</sup> class, or complete absence of teeth was noted. Such data are directly related to the DMF index and confirm the increase in dentitions defects in elderly patients. The highest OHI-S index was found in group III ( $2.33 \pm 0.14$ ), while in groups I and II the index was ( $0.92 \pm 0.13$ ) and ( $1.20 \pm 0.15$ ), respectively. Such values characterize the satisfactory hygienic condition of the oral cavity in the examined patients in groups I and II and unsatisfactory – in group III patients. The dynamics of the index in group III of the studied could be associated with age-related changes in the body's resistance, the development of comorbid pathology, depletion of the reserve of functional systems of the body that maintain homeostasis. The deterioration of the conditions for the performance of hygienic procedures, which may be caused by the use of the bridges construction, which prevailed in this group, should not be neglected. In patients of groups IV and V, this index was not determined through the dentitions defects that did not meet the examination criteria. In the examined patients in groups I and II there was a slight degree of gingivitis according to PMA ( $1.7 \pm 0.3$  % and  $7.2 \pm 0.8$  %, respectively), in groups III and IV – the average

level ( $35.1 \pm 6.5$  % and  $23.4 \pm 4.6$  %, respectively). In patients who used removable dentures, there were signs of injury to the mucous membrane of the prosthetic bed. They complained of chewing and tactile discomfort, frequent injuries to the MMOC. In some cases, allergic reactions were observed, manifested by hyperemia, edema, hemorrhage, paresthesias of the MMOC and tongue. In this case, hyperemia may be a consequence of the vasodilating effect of NO, edema – the result of the increased vascular permeability, hemorrhage – inhibitory effect of NO on platelet aggregation and adhesion.

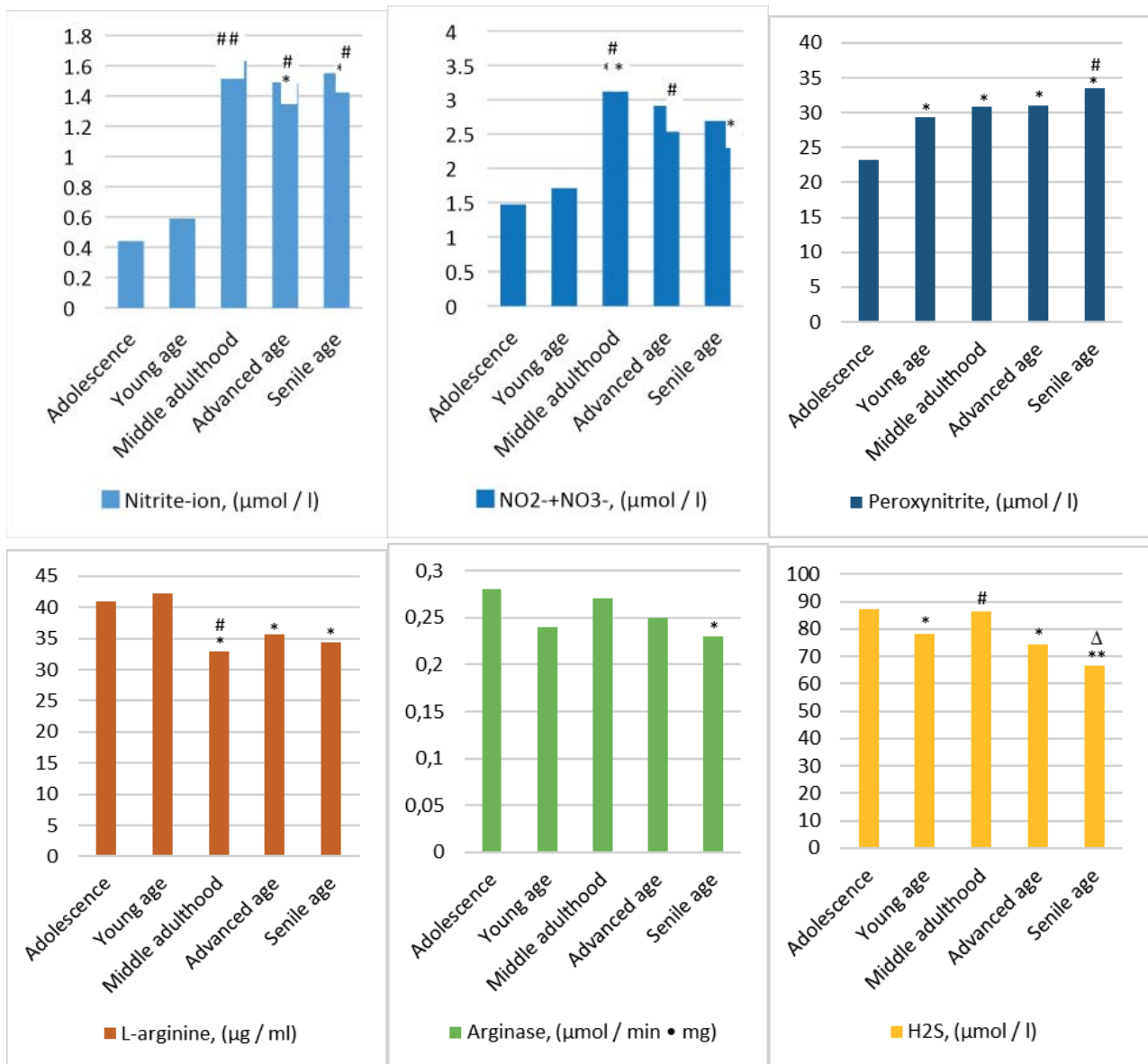
Depending on the age of the studied subjects, significant changes in the studied oral fluid biochemical parameters were revealed (Fig. 1).

The growth of NO metabolites in oral fluid with age has been determined. In particular, the content of nitrite-ion in the oral fluid of middle-aged studied patients exceeded the data in adolescents 3.7-fold ( $p < 0.01$ ), and in the advanced and senile aged people – 3.4-fold ( $p < 0.05$ ) and 3.5-fold ( $p < 0.01$ ), respectively. The same tendency was maintained regarding the amount of  $NO_2^-$  and  $NO_3^-$  in oral fluid: in the studied patients of the middle adulthood, advanced and senile age the indicator exceeded the data in the group of young aged people 2.4-fold ( $p < 0.01$ ), 2.0-fold ( $p < 0.05$ ) and 1.8-fold ( $p < 0.05$ ). Such changes occurred against the background of a gradual decrease in L-arginine and arginase activity. The concentration of peroxy-nitrite in oral fluid increased significantly in middle adulthood, exceeding the data in young people at 32 % ( $p < 0.05$ ), and in senile age – at 44 % ( $p < 0.05$ ).

Changes in the studied indices depending on the defect of the dentitions (with partial and complete loss of teeth) and their correction are represented in Table 1.

There were found significant changes in the studied parameters in patients with dentitions defect compared to the studied patients of the same age with the preserved dentitions. In particular, the levels of nitrite-ion (at 77 %,  $p < 0.05$ ), the amount of  $NO_2^-$  and  $NO_3^-$  (at 32 %,  $p < 0.05$ ) and peroxy-nitrite (at 31 %,  $p < 0.05$ ) increased in the oral fluid of the examined patients with partial loss of teeth against the background of reduced L-arginine concentrations (at 15 %,  $p < 0.05$ ) relative to the examined patients without dentitions defects. The same tendency was maintained under the conditions of complete loss of teeth according to the data in the patients with the preserved dentitions.

During the analysis of the data, the differences between the values of indices of the NO metabolism system in the examined people with partial and complete loss of teeth draw attention. Thus, an increase in the content of nitrite-ion and the sum of  $NO_2^-$  and  $NO_3^-$  under the conditions of correction with bridge or removable laminar dentures was observed at 29-53 % ( $p < 0.05$ ) relative to the corresponding values in the examined without correction



**Figure 1.** Age-related changes in the content of nitric oxide metabolism (nitrite-ion, NO<sub>2</sub><sup>-</sup> + NO<sub>3</sub><sup>-</sup>, peroxyntirite, L-arginine, arginase and hydrogen sulfide in the oral fluid of the examined patients of different age (M ± m)  
**Note:** significant differences \* p < 0.05, \*\* p < 0.01 – as for the adolescence; # p < 0.05, ## p < 0.01 – as for the young age; Δ p < 0.05 – as for the middle adulthood; ° p < 0.05 – as for the advanced age.

of dentitions. At the same time, changes in the L-arginine/arginase system were more inert. No reliable significant differences in the content of L-arginine and H<sub>2</sub>S in the oral fluid of the group of studied patients with partial loss of teeth without correction and under the conditions of correction with bridges or removable plastic dentures were found. Similarly, with complete loss of teeth, the use of complete removable laminar dentures did not significantly affect the studied parameters. However, it is important that materials with different physicochemical properties are used to make dentures. The interaction between the metals that make up the alloy or the monomer that is a component of plastics, and the oral

fluid may be accompanied by the development of chemical reactions. Therefore, even minor changes can cause metabolic disorders of oral tissues, affect the biochemical composition of oral fluid, spatial structure and activity of its components, change the acid-base balance, cause disorders of the oral microbiocenosis, colonization by pathogenic microorganisms and the development of oxidative and nitrosoxidative stress, inflammatory lesions of periodontal tissues with their subsequent structural adjustments.

In our opinion, the indices of NO and H<sub>2</sub>S significantly depend on age and dental status, because partial tooth loss was most often observed in middle-aged people,

**Table 1. Changes in the indices of metabolism of nitric oxide, L-arginine/arginase and hydrogen sulfide in the oral fluid of the examined patients with partial and complete loss of teeth and under the conditions of correction (M±m)**

Examined groups	NO <sub>2</sub> <sup>-</sup> , μmol/l	NO <sub>2</sub> <sup>-</sup> + NO <sub>3</sub> <sup>-</sup> , μmol/l	Peroxyinitrite, μmol/l	L-arginine, μg/ml	Arginase, μmol/min• mg	H <sub>2</sub> S, μmol/l
Intact dentitions	0.64 ± 0.09	1.55 ± 0.26	23.82 ± 1.02	43.01 ± 2.85	0.28 ± 0.05	87.48 ± 2.93
Partial loss of dentition:						
without correction;	1.13 ± 0.15*	2.05 ± 0.12*	31.16 ± 1.55*	39.49 ± 1.86	0.25 ± 0.08	86.11 ± 2.15
correction with bridges;	1.47 ± 0.33*	2.64 ± 0.44*	31.57 ± 2.37*	36.35 ± 0.82*	0.23 ± 0.05	88.38 ± 2.91
correction with removable laminar prostheses.	1.71 ± 0.18*#	3.13 ± 0.21*#	28.79 ± 1.98*	34.61 ± 2.16*	0.25 ± 0.06	76.39 ± 3.06*#Δ
Complete loss of teeth:						
without correction;	1.56 ± 0.15**	3.26 ± 0.27*	29.44 ± 1.76*	37.22 ± 2.33	0.24 ± 0.04	69.11 ± 2.66*#Δ
correction with complete removable laminar prostheses	1.68 ± 0.24*	3.03 ± 0.39*	35.41 ± 1.44*#Δ	35.75 ± 2.05*	0.22 ± 0.03	72.90 ± 2.55*#Δ

Note: Significant differences \*p < 0.05, \*\*p < 0.01 – as for the intact dentitions; #p < 0.05 – regarding partial loss of dentitions without correction; Δp < 0.05 – on the correction of the defect of the dentitions with bridges; \*p < 0.05 – as for the correction of the dentitions defect with the removable laminar dentures; p < 0.05 – regarding the complete loss of teeth without correction.

and complete – mainly in the advanced and senile age. In addition, the dental status (including the studied biochemical parameters of the oral fluid) is likely to be affected by changes in metabolism, bioavailability of NO, redistribution of its metabolites between individual organs and systems (including cardiovascular, digestive, excretory), the presence of concomitant pathology, taking into account the preclinical stages of their development.

It is known that NO is formed in the organism from L-arginine during NO-synthase reactions. Enzymatic or non-enzymatic oxidation of NO produces NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup>, which can also enter the body exogenously (with food or water) [9]. In this case, nitrate (nitrite)-reducing system is able to reduce them up to NO. These reactions mainly occur under conditions of hypoxia. An important part of the supply of NO to the body is the bacterial microflora of the oral cavity and gastrointestinal tract. Facultative anaerobic bacteria of the oral cavity reduce the NO<sub>3</sub><sup>-</sup> of the oral fluid up to NO<sub>2</sub><sup>-</sup>, which increase gastric NO generation in an acidic environment [9].

The sharp increase in NO concentration, which we've found in the oral fluid of the studied patients with age, reflects the increase in oxidative stress. The interaction of NO with the superoxide-anion-radical, which is accompanied by the formation of toxic peroxyinitrite, is

dangerous [8]. It was determined that the concentration of peroxyinitrite in oral fluid increased significantly in the studied people of middle adulthood, which can be used as a marker of preclinical oxidative disorders. In general, it was found that in older age groups there was an increase in the level of NO metabolites against the background of a decrease in the concentration of H<sub>2</sub>S in the oral fluid. Taking into account that H<sub>2</sub>S is involved in the regulation of NO levels (reduces its concentration), the results obtained naturally reflect individual parts of the metabolic process. Attention is drawn to the decrease in the concentration of L-arginine in the studied patients, especially in people of age groups III, IV and IV. This may indicate an increase in the formation of NO from L-arginine under the action of NO-synthase due to the inflammatory process in the tissues of the prosthetic bed and/or a good sanitation of the oral cavity. The biochemical mechanism that reduces the activity of oral arginase, which increases with age, may be the necessity to fight infection and stimulate the immune response, which increases in periodontal lesions of the oral cavity. The decrease in L-arginine may be due to the increased transport of L-arginine from oral fluid to the affected areas of periodontal soft and hard tissues and its use by inducible NO-synthase (iNOS) as a result of the increased

expression of the latter and transport membrane protein, which provides income and bioavailability of L-arginine for cells.

The results of the age dynamics of  $H_2S$  deserve special attention. Thus, the presence of  $H_2S$  in oral fluid is associated with the metabolism of sulfur-containing compounds, the sources of which are amino acids such as taurine, cysteine, homocysteine and their derivatives [14].  $H_2S$  is involved into the regulation of various metabolic processes, neurotransmission, regulation of vascular tone, maintenance of immunity, provides cytoprotective effect on the digestive tract, as well as acts as a scavenger of free radicals and antioxidants. Therefore, a decrease in the concentration of  $H_2S$  in the oral fluid with age may be accompanied by a decrease in the resistance of periodontal tissues to the aggression of oxidative (including glutamate-induced) and nitrooxidative stress. At the same time, changes in the concentration of  $H_2S$  in oral fluid reflected the dynamics of OHI-S index.

Correlation analysis showed a close inverse relationship between  $H_2S$  and peroxynitrite concentrations in oral fluid ( $r = -0.79$ ,  $p < 0.01$ ). It was found that the intensity of caries is significantly affected by the sum of  $NO_2^-$  and  $NO_3^-$  ( $r = 0.78$ ,  $p < 0.05$ ) and the concentration of peroxynitrite ( $r = 0.55$ ,  $p < 0.05$ ).

### Conclusion

1. Age-associated changes in oral fluid depend on dental status and are characterized by the increased nitrite-ion (in 3.4-3.7-fold) and markers of its endogenous synthesis of the sum of  $NO_2^-$  and  $NO_3^-$  (in 1.8-2.3-fold), increased peroxynitrite formation (at 32-44 %) against a background of a decreased L-arginine (at 13-19 %) and  $H_2S$  (at 15-24 %), inhibition of arginase activity in the middle adulthood, advanced and senile age as for the adolescence. Such changes characterize the development of oxidative and nitrooxidative stress, are the risk factors for the impaired blood supply to soft and hard periodontal tissues, followed by the formation of functional and structural changes, the development of generalized periodontitis. The results obtained are confirmed by the presence of close correlations between  $H_2S$  and peroxynitrite concentrations (inverse, strong), caries intensity and: total nitrite and nitrate levels (direct, strong), peroxynitrite content (direct, medium strength).

2. With age, the body's defense abilities are reduced, and the dental-maxillary system becomes more vulnerable to harmful factors. Metabolism of NO of the oral fluid can be influenced by local factors (inflammatory process, acidic reaction of the oral fluid, microflora imbalance, concomitant diseases of the digestive, excretory and cardiovascular systems). The studied biomarkers of oral fluid depend more on age, hygienic condition of the oral cavity, intensity of dental caries and defects of dentitions

than types of dentures. Therefore, age-related changes in the biochemical composition of the oral fluid are likely to be a trigger for structural-functional disorders of the dentomaxillofacial system.

3. The obtained data allow to recommend this non-invasive technique (determination of nitrite-ion content in the oral fluid, the amount of  $NO_2^-$  and  $NO_3^-$ , peroxynitrite,  $H_2S$  and arginase activity) to justify the indices for the performance of the prophylactic measures to prevent the development, progression and correction of periodontal disorders at the preclinical stage of the pathological process development. The list of measures should be aimed at the achievement of a balance in the system of synthesis and metabolism of NO.  $H_2S$  donors, as protectors of the inflammatory process and age-related metabolic changes, can be used in the formation of medicines and hygienic products, including those for prophylactic purpose.

**Financial Disclosure.** The study is a fragment of the research project "Complex morphofunctional research and substantiation of modern technologies application for treatment and prevention of dental diseases" (state registration No 0121U109242).

**Conflict of Interests.** The authors stated no conflict of interest.

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Received: 11.05.2022

Revised: 25.05.2022

Accepted: 26.05.2022