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“Kynurenine switch” and obesity

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ABSTRACT

Aim. To assess the concentrations of bacterial and eukaryotic metabolites mainly involved in indole, kynurenine, and serotonin pathways of tryptophan metabolism in a cohort of patients with obesity.

Materials and methods. Using high-performance liquid chromatography with mass spectrometric detection, the concentrations of several serum metabolites, such as kynurenine, kynurenic acid, anthranilic acid, xanthurenic acid, quinolinic acid, 5-hydroxyindole-3-acetate, tryptamine, serotonin, indole-3-lactate, indole-3-acetate, indole-3-butyrate, indole-3-carboxaldehyde, indole-3-acrylate, and indole-3-propionate, were analyzed in a cohort of obese patients compared with healthy volunteers.

Results. It was found that serum levels of tryptophan metabolites of microbial and eukaryotic origin were significantly increased in obese patients. Therefore, the concentration of kynurenine in the blood serum in obese patients was 2,413 ± 855 nmol / l, while in healthy volunteers of the same age group, the level of kynurenine in the blood serum was 2,122 ± 863 nmol / l. In obese patients, two acids formed due to kynurenine metabolism; the concentrations of kynurenic and quinolinic acids were increased in the blood serum. The concentration of kynurenic acid in the blood serum in obese patients was 21.1 ± 9.26 nmol / l, and in healthy patients, it was 16.8 ± 8.37 nmol / l. At the same time, the level of quinolinic acid in the blood serum in obese patients was 73.1 ± 54.4 nmol / l and in healthy volunteers – 56.8 ± 34.1 nmol / l. Normally, the level of quinolinic acid is 3.4 times higher than the concentration of kynurenic acid, and in case of obesity, there is a comparable increase in these acids in the blood serum.

From indole derivatives, mainly of microbial origin, the concentrations of indole-3-lactate, indole-3-butyrate, and indole-3-acetate were significantly increased in the blood serum of obese patients. In obese patients, the serum concentration of 5-hydroxyindole-3-acetate was elevated to 74.6 ± 75.8 nmol / l (in healthy volunteers – 59.4 ± 36.6 nmol / l); indole-3-lactate – to 523 ± 251 nmol / l (in healthy volunteers – 433 ± 208 nmol / l); indole-3-acetate – to 1,633 ± 1,166 nmol / l (in healthy volunteers – 1,186 ± 826 nmol / l); and indole-3-butyrate – to 4.61 ± 3.31 nmol / l (in healthy volunteers – 3.85 ± 2.51 nmol / l).

Conclusion. In case of obesity, the utilization of tryptophan was intensified by both the microbiota population and the macroorganism. It was found that obese patients had higher concentrations of kynurenine, quinolinic and kynurenic acids, indole-3-acetate, indole-3-lactate, indole-3-butyrate, and 5-hydroxyindole-3-acetate. Apparently, against the background of increased production of proinflammatory cytokines by adipocytes in obese patients,

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the “kynurenine switch” was activated which contributed to subsequent overproduction of tryptophan metabolites involved in the immune function of the macroorganism.

Key words: microbiota, tryptophan, obesity, kynurenines, indoles, metabolic syndrome.

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Conformity with the principles of ethics. All participants signed an informed consent to take part in the study. The study was approved by the local Ethics Committee at Pirogov Russian National Research Medical University (Protocol No. 186 of 26.06.2019).

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«Кинурениновый переключатель» и ожирение

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РЕЗЮМЕ

Цель. Изучить содержание метаболитов бактериального и эукариотического происхождения индольного, кинуренинового и серотонинового путей обмена триптофана у пациентов с ожирением.

Материалы и методы. Методом высокоэффективной жидкостной хроматографии с масс-спектрометрическим детектированием изучили концентрации сывороточных метаболитов: кинуренина, кинуреновой кислоты, антраниловой кислоты, ксантуреновой кислоты, хинолиновой кислоты, 5-гидросииндол-3-ацетата, триптамина, серотонина, индол-3-лактата, индол-3-ацетата, индол-3-бутирата, индол-3-карбоксальдегида, индол-3-акрилата, индол-3-пропионата у пациентов с ожирением в сравнении с группой здоровых добровольцев.

Результаты. Установлено, что у пациентов с ожирением в сыворотке крови статистически значимо повышен уровень метаболитов триптофанового обмена микробиотического и эукариотического происхождения. Концентрация кинуренина в сыворотке крови у больных с ожирением составляла 2 413 ± 855 нмоль/л, тогда как у здоровых добровольцев такой же возрастной группы – 2 122 ± 863 нмоль/л. Также у пациентов с ожирением в сыворотке крови были повышены две кислоты, которые образуются в результате метабо-

лизма кинуренина – кинуреновая и хинолиновая. Концентрация кинуреновой кислоты в сыворотке крови у пациентов с ожирением составляла 21,1 ± 9,26 нмоль/л, а у здоровых 16,8 ± 8,37 нмоль/л соответственно. Тогда как концентрация хинолиновой кислоты в сыворотке крови при ожирении – 73,1 ± 54,4 нмоль/л, а у здоровых добровольцев – 56,8 ± 34,1 нмоль/л. В норме концентрация хинолиновой кислоты в 3,4 раза выше, чем концентрация кинуреновой кислоты, а при ожирении происходит сопоставимое их повышение.

Из производных индола, которые имеют преимущественно микробиотическое происхождение, в сыворотке крови пациентов с ожирением статистически значимо повышена концентрация индол-3-лактата, индол-3-бутирата и индол-3-ацетата. У пациентов с ожирением концентрация в сыворотке крови метаболита серотонина – 5-гидроксииндол-3-ацетата – была повышена и составляла 74,6 ± 75,8 нмоль/л (у здоровых добровольцев – 59,4 ± 36,6 нмоль/л); индол-3-лактата – 523 ± 251 нмоль/л (у здоровых добровольцев 433 ± 208 нмоль/л); индол-3-ацетата – 1 633 ± 1166 нмоль/л (у здоровых добровольцев 1 186 ± 826 нмоль/л); индол-3-бутирата – 4,61 ± 3,31 нмоль/л (у здоровых добровольцев 3,85 ± 2,51 нмоль/л).

Заключение. При ожирении происходит интенсификация утилизации триптофана как микробиотической популяцией кишечника, так и макроорганизмом. Установлено, что больные с ожирением имеют более высокие концентрации кинуренина, хинолиновой и кинуреновой кислот, индол-3-ацетата, индол-3-лактата, индол-3-бутирата и 5-гидроксииндол-3-ацетата. Видимо, на фоне гиперпродукции провоспалительных цитокинов адипоцитами у пациентов с ожирением срабатывает «кинурениновый переключатель», что и обеспечивает гиперпродукцию метаболитов триптофанового обмена, которые вовлечены в иммунологическую функцию макроорганизма.

Ключевые слова: микробиота, триптофан, ожирение, кинуренины, индолы, метаболический синдром.

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INTRODUCTION

Tryptophan is an essential amino acid for the human body [1]. However, the intestinal microbiota has an enzymatic system of the shikimate pathway of tryptophan synthesis and can provide the macroorganism with both amino acid and its derivatives. Among these metabolites are derivatives of the kynurenine, indole, and serotonin pathways of tryptophan metabolism [2]. Synthesis of aromatic amino acids is possible in the microbiome due to universal metabolic pathways – glycolysis and the pentose phosphate pathway (PPP). Therefore, glycolysis produces phosphoenolpyruvate (PEP), while PPP is a source of erythrose–4-phosphate for the shikimate pathway of tryptophan and anthranilic acid synthesis (Fig. 1,*a* and *b*).

The dominant mechanism of tryptophan utilization for eukaryotes is the kynurenine pathway. More than 90% of tryptophan not spent on biosynthesis is converted into metabolites of the kynurenine pathway [2]. They perform antioxidant and anti-inflammatory functions in the body, regulate immune responses, and act as toxins and signaling molecules in the molecular dialogue between the macroorganism and the microbiome [2]. For example, anthranilic acid (anthranilate) is an essential link in the metabolic coupling of the microbiome and the macroorganism: it has a mixed origin and is a precursor in the synthesis of quinolinic acid and, accordingly, NAD^+ and also participates in the formation of quinoline regulators of the quorum sensing (QS) in the microbiome [3]. So far, there is no information on the role

of microbiota enzymes in the synthesis of quinolinic acid and NAD^+ directly from tryptophan [4]. However, both quinolinic acid and NAD^+ are not species-specific metabolites, and their synthesis occurs in both eukaryotes and prokaryotes. It is important to note that quinolinic acid in eukaryotes is formed from tryptophan, whereas in prokaryotes, this acid is formed from aspartic acid [5].

Quinolinic acid is neuro- and gliotoxic and increases in animals with multiple sclerosis in brain tissues and blood serum [6]. Quinolinic acid is an ionotropic glutamate receptor antagonist that selectively binds N-methyl-D-aspartate, while kynurenic acid is an agonist of glutamatergic and cholinergic receptors and has antioxidant properties [7]. Therefore, kynurenic acid will have a neuroprotective effect.

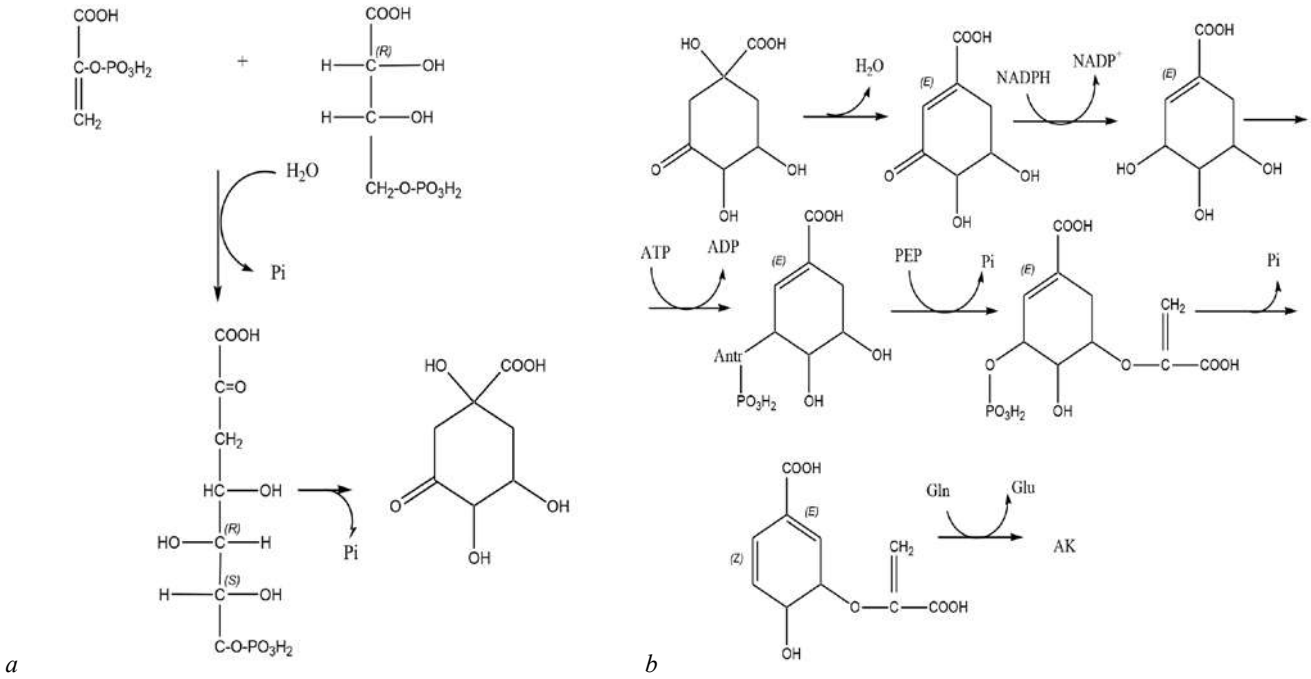

Fig. 1. The shikimate pathway: *a* – at the first stage, 3-dehydroquinate is synthesized: PEP and erythrose 4-phosphate are converted into deoxy-d-arabino-hept-2-ulosonate-7-phosphate (DAHP) with the participation of DAHP synthase; *b* – at the second stage, DAHP is converted into 3-dehydroquinate (3-DQ) with the participation of 3-DQ synthase, and then reduction to shikimate occurs, followed by a phosphorylation reaction to shikimate 3-phosphate. When interacting with PEP, shikimate 3-phosphate is converted into enolpyruvate shikimate-3-phosphate by a specific synthase, and then, after dephosphorylation, it becomes a chorismate, which, when interacting with glutamine (Gln), turns into anthranilic acid (AA)a

It is shown that after tryptophan loading or after immune stimulation, hepatocytes, which are constitutively responsible for NAD^+ synthesis, transiently accumulate quinolinic acid. At the same time, cells of the immune system, including macrophages, dendritic cells, Langerhans cells, Kupffer cells, etc., generate high, stable levels of intracellular quinolinic acid in response to various immune stimulators. This event regulates the mobility of immune cells, since it induces synthesis of cytoskeleton proteins in them [8]. It is important to note that in the liver, all tryptophan molecules not involved in protein synthesis are converted into NAD^+ or oxidized to CO_2 and H_2O. During immune stimulation, indolamine-2,3-dioxygenase (I-2,3-DO) is activated in the lung tissue, contributing to the "kynurenine switch" activation. Systemic kynurenine begins to actively engage immune cells for overproduction of NAD^+ [8].

Intracellular levels of quinolinic acid increase in response to immune stimulation by lipopolysaccharide in macrophages, microglia, dendritic cells, and other cells of the immune system [8]. The further fate of quinolinic acid depends on the activity of quinolinate phosphoribosyltransferase (QPRT), which catalyzes the formation of nicotinic acid mononucleotide from quinolinic acid and 5-phosphoribosyl-1-pyrophosphate (Fig. 2).

It is known that the inflammatory response requires higher levels of NAD^+ in immune cells. Thus, NAD^+ performs numerous functions (Fig. 2):

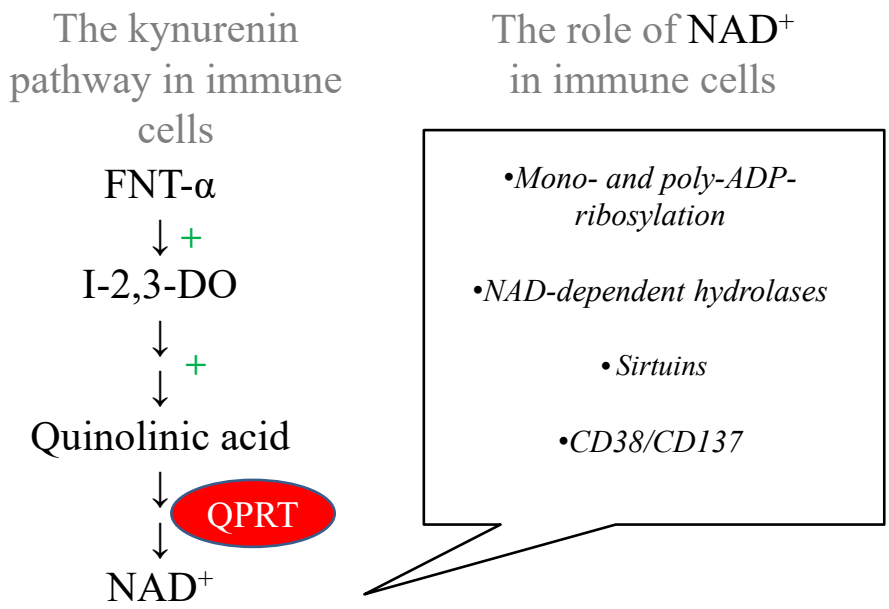

Fig. 2. The biological role of NAD+ in immune cells

participates in the respiratory burst and the polymerization reaction of poly(ADP-ribose) polymerase (which is involved in DNA repair mechanisms) and regulates the activity of NAD^+-dependent deacetylases (sirtuins) and NAD^+-dependent hydrolases, including CD38, CD157, and ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase. The latter is expressed on many immune cells, including $CD4^+$, $CD8^+$, B-lymphocytes and natural killer cells, and CD157 – ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase-2 is expressed by pre-B cells [8].

It was shown that obesity is a systemic inflammatory disease with overexpression of indoleamine 2,3-dioxygenase (I-2,3-DO) in both white adipose tissue and the liver, induced by adipocytes of proinflammatory cytokines [9]. In response to the activation of the regulatory enzyme of the kynurenine pathway, the entire kynurenine pathway of tryptophan metabolism is also intensified. Activation of I-2,3-DO in the intestine leads to an increase in the kynurenine concentration, which has an antimicrobial effect [2] and, thus, affects the gut microbiota.

It is possible that in obesity, the intensification of the shikimate pathway in the intestine is accompanied by excessive production of tryptophan, which can be metabolized into indole derivatives by various microbiota populations. It should be noted that indole-3-lactate regulates the kynurenine pathway of tryptophan conversion in cells of the macroorganism [10], and indole-3-acetate (and tryptamine) is an agonist of aryl hydrocarbon receptors [11].

Therefore, the aim of the study was to investigate the concentrations of metabolites of the kynurenine and indole pathways of tryptophan metabolism in the blood serum of patients with obesity.

MATERIALS AND METHODS

A cohort of 266 participants with an average age of 39.9 ± 4.2 years was examined. Two clinical groups were formed. Group 1 (n = 138) – a control group of healthy volunteers without obesity and/or metabolic syndrome with an average body mass index (BMI) of 22.7 kg / m^2 and waist circumference (WC) of 79.8 cm. Group 2 (n = 128) was an experimental group including patients with obesity and/or metabolic syndrome with an average BMI of 32.96 kg / m^2 and WC of 108.98 cm.

Samples of the whole blood and blood serum were obtained from all study participants according to the study protocol. Transportation and storage of the samples were carried out in compliance with the cold chain at a temperature not higher than –40 °C.

A quantitative analysis of tryptophan metabolites in the blood serum was performed by high-performance liquid chromatography with mass spectrometry (HPLC-MS/MS). The analysis was performed using an Agilent 1200 liquid chromatography (Agilent Inc., USA) with an automatic sample input system, a

column thermostat, and a degasser. Chromatographic separation was performed using the Discovery PFP HS F5 analytical column (2.1 × 150 mm; 3 microns). The composition of the mobile phase A – 0.1% formic acid solution in deionized water; phase B – 100% acetonitrile for chromatography. The mobile phase gradient ranged from 1 to 10 % within 4 minutes, then went up to 90% by the 9th minute of the analysis. The flow rate of the mobile phase was 0.40 ml / min.

A mass spectrometry detector based on the Agilent 6460 triple quadrupole (Agilent Inc., USA), MRM, and electrospray ionization were used. The characteristic parent and daughter ions for each compound for the MRM mode and ionization and dissociation parameters were optimized using the standards of the studied metabolites. The received signal was processed using MassHunter software (Agilent Inc., USA).

Metabolite concentrations were calculated by the internal standard method (2-hydroxynicotinic acid). The internal standards of the determined compounds were prepared using an artificial matrix containing bovine serum albumin and sodium chloride. The studied metabolites were added to the matrix and prepared according to the analysis method.

Serum sample preparation was conducted as follows: an internal standard (2-hydroxynicotinic acid) was added to 100 µl of serum, and proteins were precipitated with acetonitrile; the supernatant was evaporated and re-dissolved in 10% methanol in water with the addition of ascorbic acid to prevent oxidation of analytes. To prepare a stool sample, it was lyophilized to a dry residue, and then a sample of about 5 mg was extracted with 50% methanol in water with addition of an internal standard and ascorbic acid. After centrifugation, the sample was analyzed by HPLC-MS/MS.

The method was validated in terms of selectivity, linearity, accuracy, reproducibility, matrix effect, and analyte stability. The validation was conducted following the FDA guidelines for the validation of bioanalytical methods.

Statistical analysis of the obtained results was performed using Statistica 12.0 software package (StatSoft Inc, USA). The data are presented as the mean and standard deviation $M \pm \sigma$. After checking the data distribution for normality, the statistical significance of the differences in the mean values of independent samples was assessed using parametric analysis. The normality of the distribution of variables in the groups was assessed using the Shapiro – Wilk test. The Mann – Whitney test was used for comparative analysis of independent samples, with the Wilcoxon test used for comparative analysis of dependent samples. The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

When analyzing the level of metabolites of the kynurenine and indole pathways in the blood serum in the control group, we found that concentrations of quinolinic acid, kynurenic acid, kynurenine, 5-hydroxyindole-3-acetate, indole-3-lactate, indole-3-acetate, and indole-3-butyrate significantly increased in obesity (Table).

Table

Serum content of tryptophan metabolites, nmol/l, $M \pm \sigma$

Parameter	Control group, $n = 138$	Experimental group, $n = 128$
Quinolinic acid	56.8 ± 34.1	73.1 ± 54.4*
Kynurenine	2,122 ± 863	2,413 ± 855**
5-hydrosyindole-3-acetate	59.4 ± 36.6	74.6 ± 75.8*
Kynurenic acid	16.8 ± 8.37	21.1 ± 9.26*
Indole-3-lactate	433 ± 208	523 ± 251**
Indole-3-acetate	1,186 ± 826	1,633 ± 1,166*
Indole-3-butyrate	3.85 ± 2.51	4.61 ± 3.31*
Serotonin	809 ± 356	782 ± 434
Anthranilic acid	33.3 ± 20.7	37.5 ± 20.9
Xanthurenic acid	4.31 ± 3.11	4.18 ± 2.91
Tryptamine	0.818 ± 0.541	0.731 ± 0.314
Indole-3-carboxaldehyde	40.4 ± 19.3	44.3 ± 25.7
Indole-3-acrylate	5.01 ± 14.6	4.29 ± 15.7
Indole-3-propionate	650 ± 845	753 ± 736

* differences are statistically significant relative to the control group at $p < 0.01$; ** differences are statistically significant relative to the control group at $p < 0.05$.

We did not find statistically significant differences in serum concentrations of such tryptophan metabolites as anthranilic acid, xanthurenic acid, tryptamine, indole-3-carboxaldehyde, indole-3-acrylate, and indole-3-propionate between healthy volunteers and obese patients (Table).

Many research groups demonstrated an increase in kynurenine concentration in the blood serum in obesity [4]. However, in this study, the statistically significant increase in serum kynurenine was not so significant in percentage terms, whereas we found a tremendous increase in the concentrations of quinolinic acid and kynurenic acid in obese patients.

We also showed that among all the kynurenine pathway metabolites in the blood serum in both groups, the concentration of kynurenine was significantly higher in comparison with other metabolites of the kynurenine pathway. In our opinion, this observation is natural, since kynurenine is the main catab-

olite of tryptophan for the macroorganism. However, kynurenine has a mixed origin, and it remains unclear what percentage of serum kynurenine is absorbed from the intestine and what percentage is formed in other organs of the macroorganism in normal conditions and obesity.

The depletion of taxa in the gut microbiota in obese patients is known [12]. Overproduction of various proinflammatory cytokines by adipocytes is also observed [13], accompanied by an increase in the kynurenine concentration in the blood serum with a subsequent rise in quinolinic acid. The increased concentration of quinolinic acid in the blood serum of obese patients reflects what is happening in the cells of the macroorganism. The significance of the "kynurenine switch" and the protective role of NAD^+ were discussed above, but there is a rate-limiting reaction in the synthesis of NAD^+ – CPRT [8]. Apparently, overproduction of quinolinic acid in obese patients is associated with low activity of this enzyme, also in immune cells.

Utilization of the excessive amount of kynurenine in obesity also follows the path of its conversion into kynurenic acid, as evidenced by data obtained on a statistically significant increase in the level of kynurenic acid in obese patients. This metabolite performs an antioxidant role and is seemingly a functional antagonist of quinolinic acid, not only in the nervous tissue. Probably that is why the increase in the serum concentration of quinolinic acid is comparable to the increase in the serum level of kynurenic acid in obese patients.

A pronounced statistically significant increase in the level of indole-3-acetate in the blood serum was found in the patients of the experimental group. This tryptophan metabolite is mainly of bacterial origin. It should be noted that the indole molecule itself suppresses the formation of *Pseudomonas* quinolone signal (PQS) in the intestine [14], the precursor of which is anthranilic acid.

It was shown that indole-3-acetate is a metabolite conjugating the indole and kynurenine tryptophan metabolism pathways; it can also be formed in eukaryotic cells from anthranilic acid. This metabolite is a ligand of aryl hydrocarbon receptors and performs a regulatory function in the human body, participating in the immune function [15]. Indole-3-lactate is also an agonist of aryl hydrocarbon receptors [16]. It is known that indole-3-lactate decreases the response to interleukin-8 after stimulation with interleukin-1 [17]. In obese patients, an increase in indole-3-lactate is microbiota metabolic compensation to reduce the production of proinflammatory cytokines. In further studies, it is necessary to analyze the relationship between the concentration of proinflammatory cytokines and indole-3-lactate in obese patients.

Indole-3-butyrate, like indole-3-lactate, is a metabolite of bacterial origin. So far, there are no studies that would show the eukaryotic origin of these metabolites of the indole pathway in tryptophan metabolism. The involvement of indole-3-butyrate in the pathogenesis of obesity has to be studied, while there are no studies that would determine the role of this tryptophan metabolite. We found that in obese patients, the concentration of indole-3-butyrate in the blood serum was higher than in healthy volunteers.

When serotonin is metabolized, 5-hydroxyindole-3-acetate is formed, which is significantly increased in the blood serum of obese patients, while the serum concentration of serotonin itself does not change significantly. It should be noted that the primary level of serum serotonin is determined by its overproduction in enterocytes. It can be assumed that an excessive amount of tryptophan in the intestine is metabolized to serotonin, indole-3-acetate, indole-3-butyrate, indole-3-lactate, and kynurenine.

CONCLUSION

It should be noted that all pathways of tryptophan metabolism in the body are intensified in obese patients. Utilization of the excessive amount of tryptophan occurs by both gut microbiota and cells of the macroorganism. An increase in the serum level of quinolinic acid has an adverse effect on the macroorganism and is a necessary consequence of immune stimulation. An increase in the concentration of kynurenic acid in the blood serum of patients with low-grade inflammation in obesity is an indispensable condition for simultaneous formation of a functional antagonist of quinolinic acid.

In obese patients, we have found an increase in the rate of tryptophan metabolism by the gut microbiota, and it has not been studied yet whether this tryptophan is of exogenous origin or a product of the shikimate pathway. The increased levels of kynurenine, quinolinic acid, and kynurenic acid in the blood serum may be a manifestation of the "kynurenine switch" of NAD^+ overproduction in immune cells in obese patients. A drastic increase in indole-3-acetate in the blood serum of obese patients performs a compensatory and adaptive function, also through suppression of the activity of some enzymes of the kynurenine pathway in different tissues of the macroorganism. The

increase in the concentrations of indole-3-lactate and indole-3-butyrate in the blood serum of obese patients reflects precisely the microbiotic activation of the utilization of excess tryptophan, possibly of shikimate origin. In obese patients, the utilization of serotonin is intensified, and the concentration of 5-hydroxyindole-3-acetate in the blood serum increases statistically significantly.

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Authors contribution

Shestopalov A.V. – design of the study, compilation of a database, revision of the article, writing of all the sections, final approval of the manuscript for publication. Shatova O.P. – selection of the patient sample, review of publications of the topic of the study, statistical analysis of data and drafting of the article, compilation of tables and figures, final proofreading of the manuscript. Karbyshev M.S. – writing of the "Discussion" section, writing of the article in English. Gaponov A.M. – design of the study, writing of the "Materials and methods" section. Moskaleva N.E. – carrying out of the biochemical analyses, discussion of the obtained results. Appolonova S.A. – carrying out of the biochemical analyses, editing of the "Materials and methods" section. Tutelyan A.V. – critical revision of the manuscript for important

intellectual content. Makarov V.V. – discussion of the study design and the obtained data. Yudin S.M. – editing of the study concept, interpretation of the research results. Roumiantsev S.A. – design of the study, revision of the article, drafting of all the sections, final approval of the manuscript for publication.

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