VIOLATION OF MICROBIAL AND ENDOGENOUS METABOLISM IN CELIAC DISEASE

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Abstract: Aim: to investigate a fecal microbiota composition and to identify candidate biomarkers of celiac disease (CD) by serum metabolomics analysis.
Methods: the quantitative real-time polymerase chain reaction was used for fecal microbiota assessment. Serum metabolomic assays were conducted using the GC–MS.
Results: serum of CD patients showed significant increases in stearic acid, 2-HIVA, succinate, fumarate and benzoate compared to HC. A decrease in the level of eicosadiene and an increase in AA in blood were determined. The ratio of AA to EDA was statistically significant (4.84 vs. 3.28, p=0.033). The elongase activity index in patients with celiac disease tended to increase (p=0.067). The colon microbiome in CD was characterized by decreasing in the level of butyrate-producing Faecalibacterium prausnitzii (F.p.) and Bifidobacterium spp.. Significant negative correlations were observed; between the levels of Bifidobacterium spp. and F.p. and the concentration of succinic acid (rs= =–0.343 [p=0.026] and rs=–0.430 [p=0.005], respectively); the F.p. and the fumaric acid (r=–0.429, p=0.005); the benzoic acid and the amount of Bifidobacterium spp. (r=–0.341, p=0.025).
Conclusion: significant changes in serum levels of microbial and endogenous metabolites, reflecting some metabolic pathways disturbances were observed in CD. Metabolites and metabolomic index reflecting the balance between pro-inflammatory and anti-inflammatory components, may be considered as candidate biomarkers of chronic inflammation and metabolic dysbiosis in CD. An increased B. fragilis/F.p. ratio can serve as available biomarker for intestinal pro-inflammatory dysbiosis in CD.
Keywords: butyrate, butyric acid, celiac disease, dysbiosis, Faecalibacterium prausnitzii, gut microbiota, serum metabolome, biomarkers, metabolomics.

1. Introduction

Modern experimental and scientific data support the hypothesis that immune-mediated inflammation is closely related to intestinal microbiota changes in celiac disease [1–3]. Celiac disease is a chronic genetically determined inflammatory disease, the target organ in which are enterocytes of the mucous membrane (MM) of the small intestine. The leading role in the development of celiac disease is played by pathological autoimmune reactions that cause multisystem damage [4]. The relevance of the intestinal barrier dysfunction in the development of chronic inflammation of the small intestine has been repeatedly confirmed in scientific studies [5, 6]. The impairment MM's permeability is connected with inherited polymorphism of susceptibility genes (predispositions), such as MYO9B, PARD 3, MAGI2, DLG5 (single nucleotide polymorphism R 30Q [rs1248696]) and PTPN 2 [7, 8]. The role of pro-inflammatory cytokines involved in the pathogenesis of celiac disease in modulating the composition of microbiota (IL-15), a decrease in the number of butyrate-producing bacteria, is described. At the same time, the translocation of microorganisms promotes the activation of immune cells and enhances the production of cytokines, contributing to the chronicity of gastrointestinal inflammation. Intestinal dysbiosis in celiac disease is characterized by both taxonometric changes in the type of pro-inflammatory dysbiosis and impaired integration of microbial metabolism with human metabolism – metabolic dysbiosis [9]. It is important to note that these signs are determined both in primary patients and in patients on a gluten-free diet (GFD). In the first case, we can talk about the possible etiopathogenetic role of dysbiotic changes (primary dysbiosis), in the second – about secondary intestinal dysbiosis associated with a decrease in dietary fiber intake with a gluten-free diet [10, 11]. To modern date, about 200 metabolites of microbial origin that can act as potential biomarkers of various diseases have been identified. The level of a number of metabolites in the blood and other biological fluids is largely determined by the metabolic activity of the intestinal microbiota [12, 13]. Significant changes in the metabolism of blood, urine, feces and mucous membranes, reflecting malabsorption, impaired energy metabolism, intestinal microbiocenosis and intestinal barrier were also detected in celiac disease.

Thus, disorders of microbial metabolism are a poorly studied problem, and the determination of specific microbial metabolites is of particular interest in the development of new methods for the diagnosis of celiac disease.

Aim: to investigate a fecal microbiota composition and to identify candidate biomarkers of celiac disease (CD) by serum metabolomics analysis.

2. Materials and Methods

The study has been performing from 2015 to 2018 year on Department of Propaedeutics of internal diseases, gastroenterology and dietoligy named after I. I. Rissa of North-Western State Medical University named after I. I. Mechnikov. Fresh fecal samples were collected from 43 CD patients on a gluten-free diet and 42 healthy control (HC) patients. The characteristics of the object of study are presented in **Table 1**.

Table 1
Characteristics of the main and control groups of the study

Attribute	CD	HC
Number	43	42
Age	18–60 years	18–60 years
F/M ratio	1/1.5	1/1.5
GFD	at least for the last 6 months	–
Disease activity	remission	–

All patients who participated in the study signed documents according with the ethical standards presented in the Helsinki Declaration of the World Medical Association "Recommendations for Physicians Engaged in Biomedical Research with People" (2000).

The diagnosis of celiac disease was established on the basis of anamnesis, endoscopic, histomorphological and biochemical studies (morphometry of the mucous membrane of the retrobulbar duodenum, HLA typing, immunological blood test) [14]. The study did not include patients undergoing acute infectious diseases, as well as taking any antibacterial, antiviral, antifungal and antiprotozoal drugs, probiotics, prebiotics and drugs (dietary supplements) containing bacterial metabolites or their synthetic analogues, drugs with prokinetic activity in less than 30 days before the start of the study.

For fecal microbiota assessment, the quantitative real-time polymerase chain reaction (qRT-PCR) was used. Segregation (extraction) of DNA from evacuation samples was carried out in accordance with generally accepted standards [15]. To identify the group of butyrate-producing bacteria (BPB) by real-time PCR, the corresponding degenerate primers BCoATscrF (direct) and BCoATscrR (reverse) were used to amplify the butyryl-CoA gene: acetate-CoA transferase, the main enzyme responsible for the production of butyric acid by the microbiota of the colon. Serum metabolomic assays were conducted using the GC-MS (GCMS-QP2010 Plus, Shimadzu Corporation, Kyoto, Japan). The identification of low-molecular-weight compounds according to GC–MS was performed using the international databases NIST 05, NIST 08, Human Metabolome Database (HMDB; http://www.hmdb.ca) and Serum Metabolome database (SMDB; http://www.serummetabolome.ca) [16, 17]. The data obtained during the chromatography-mass spectrometric studies were normalized to the content of heptadecanoic acid (C 17:0), selected as the reference compound, the concentration of which was taken as 1 conventional unit.

Statistical data processing was performed using methods of the IBM SPSS Statistics 20 software (IBM Corp., USA). The normality of the data distribution was checked using the Kolmogorov – Smirnov test, with the correction of the Lilliefors test and Shapiro – Wilk test. Due to the lack of a normal distribution of data, the median (Me) was used for the description, indicating the boundaries of the interquartile range, which are the 25th (Q1) and 75th percentiles (Q3), respectively. Quantitative data were evaluated using nonparametric criteria: the Mann – Whitney U test and the Kolmogorov – Smirnov test. Hypothesis testing of the significance of differences between frequencies was conducted using the Fisher's exact test. The information content of potential biomarkers was evaluated using a Receiver Operating Characteristic [ROC] Curve analysis. The critical value of the significance level (p) was taken equal to 0.05.

3. Results

93 compounds were identified: 28 metabolites – could have a double (endogenous + microbial) or predominantly microbial origin; for analysis, 37 metabolites are applicable. Selection criteria:

1) metabolites of microbial origin;

2) metabolites possibly associated with inflammatory and autoimmune intestinal pathologies;

3) the most important endogenous metabolites (Krebs cycle, citric acid cycle).

Metabolites of the following groups were determined in blood serum: phenocarboxylic acids, hydroxycarboxylic acids, dicarboxylic and monocarboxylic acids. Only substances whose level change was statistically significant were included in the discussion.

Serum of CD patients showed significant increases in stearic acid, 2-hydroxyisovaleric acid (2-HIVA), succinate, fumarate and benzoate compared to HC (**Fig. 1**).

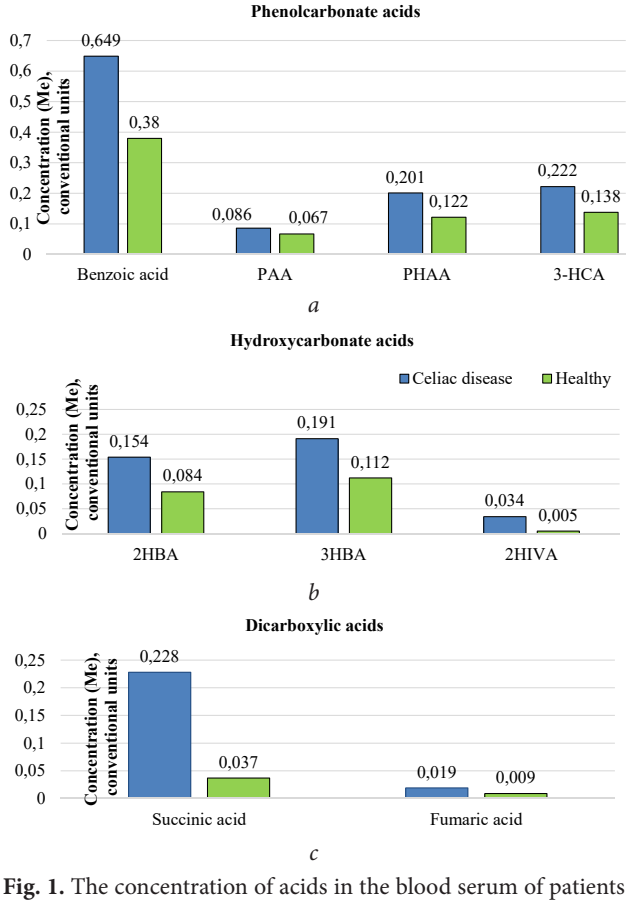

Fig. 1. The concentration of acids in the blood serum of patients with celiac disease and healthy volunteers: *a* – phenocarboxylic acids; *b* – hydroxycarboxylic acids; *c* – dicarboxylic acids; PAA – phenylacetic acid; PHAA – parahydroxyphenyl acetic acid, 3-HCA – hydroxycinnamic acid, 2HBA – 2-hydroxybutyric acid, 3HBA – 3-hydroxybutyric acid, 2HIVA-2-hydroxyisovalerate acid

Changes in the concentration of monocarboxylic acids did not have statistical significance compared with healthy volunteers. A decrease in the level of eicosadiene and an increase in arachidonic acids in blood included in the group of polyunsaturated fatty acids (ω-6-PUFA) were determined. In addition, a number of metabolic indices, biomarkers of fatty acid metabolism, were calculated. Thus, the level of increase in arachidonic acid (AA) in the blood of patients with celiac disease did not have significant differences with the control group (**Fig. 2**). However, the ratio of AA to eicosadienoic acid (EDA) (C20: 4n-6/C20:2n-6) was statistically significant (4.84 vs. 3.28, p=0.033; Mann-Whitney U-test). The elongase activity index in patients with celiac disease tended to increase, but the differences were not statistically significant (p=0.067; Mann-Whitney U-test).

Analysis of feces microflora showed that the colon microbiome of patients with celiac disease was characterized by a significant decrease in the level of butyrate-producing Faecalibacterium prausnitzii and Bifidobacterium spp. compared with healthy volunteers (p<0.005). At the same time, the total number of bac-

teria, bacterial groups of Bacteroides fragilis, Lactobacillus and Escherichia coli did not have significant differences. Statistically significant changes in microbiocenosis were accompanied by deviations of indicators of metabolites of microbial origin in blood serum, which can be interpreted as signs of metabolic dysbiosis. Thus, significant negative correlations between the levels of representatives of probiotic bacteria – Bifidobacterium spp. and Faecalibacterium prausnitzii in feces and the concentration of succinic acid in the blood serum (rs=–0.343 [p=0.026] and rs=–0.430 [p=0.005], respectively), as well as a significant negative correlation between the level of Faecalibacterium prausnitzii in feces and the concentration of fumaric acid in blood serum (r=–0.429, p=0.005), indirectly confirming the pro-inflammatory properties of succinate and fumarate. The level of benzoic acid was significantly reduced compared with the control group, and negatively correlated with the amount of Bifidobacterium spp. in feces (r=–0.341, p=0.025).

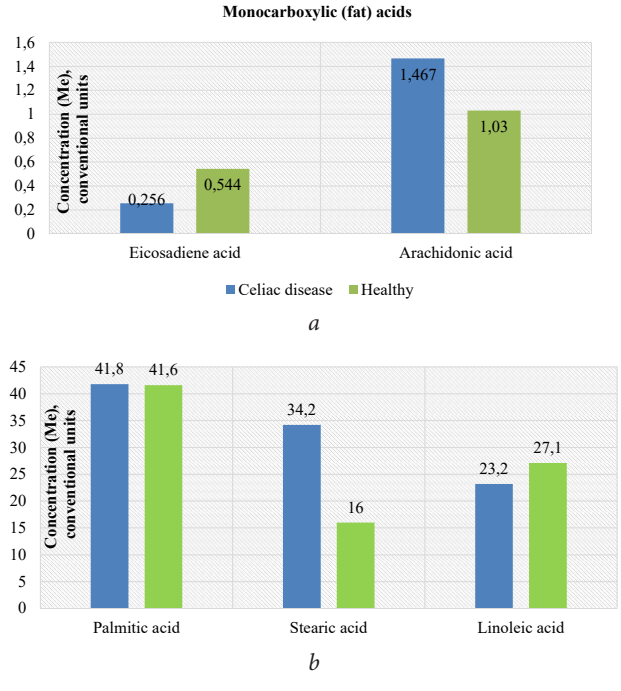

Fig. 2. The concentration of monocarboxylic acids in the blood serum of patients with celiac disease and healthy volunteers: *a* – the concentration of eicosadienoic and arachidonic acids; *b* – the concentration of palmitic, stearic and linoleic acids

3. Discussion and conclusions

Currently, carboxylic acids (food, microbial and endogenous origin) are considered as signalling molecules, regulatory and immunomodulation compounds in the human body. Mainly bacteroides and representatives of the clostridium genus produce phenocarboxylic acids, the increase of which was observed in patients with celiac disease. Therefore, Clostridium bifermentans, Clostridium difficile, Clostridium perfringens and Paeniclostridium sordellii are producers of phenylacetic acid, according to Intestinibacter bartlettii – PAA and PHPAA, and Clostridium sporogenes – phenylpropionic acid and parahydroxypropionic acid [18]. The latter, undergoing oxidation processes in the liver, become a substrate for the formation of benzoic acid. Benzoic and 3-hydroxyphenylpropionic acid are the most important microbial metabolite of caffeic acid, and are more common in patients with Clostridium difficile and Campylobacter jejuni infections [19]. The experimental results proved the role of phenylcarboxylic acids in inhibiting the growth of immune tissue of the spleen, liver and pancreas, which determines their pathogenetic role in the development of immune-mediated chronic diseases. The above makes it possible to use these acids as metabolic markers of pathologies accompanied by a disturbed immune response and chronic impaired microbial and endogenous metabolism, such as celiac disease.

In the group of hydroxycarboxylic acids, a significant increase in 2HIVA, an intermediate in the ketogenesis and metabolism of valine, leucine and isoleucine, was noteworthy. The main producers of 2HIVA are Listeria spp., Eggerthella lenta and Prevotella, whose role in impaired intestinal barrier permeability increases with bacteraemia. Thus, the results obtained may be the result of increased bacterial production of 2HIVA because of intestinal microbiocenosis disorders. An increase in the concentration of hydroxycarboxylic acids in blood compared to the control group against the background of a decrease in the intestinal butyrate-producing bacteria Faecalibacterium prausnitzii indirectly indicates the involvement of this group of metabolites in the energy supply of colonocytes. A significant increase in the concentration of succinic acid was noted in blood of the examined patients, the main producers of which are Bacteroides spp., Eggerthella lenta, Paraprevotella clara, Paraprevotella xylaniphila, Marvinbryantia formatexigens, Ruminococcus champanellensis, Enterococcus faecalis. During normal operation of the digestive tract, succinic and lactic acids are intermediate products of fermentation and do not accumulate in significant quantities, serving as co-substrates for the production of propionic and butyric acids. However, their number increases with intestinal dysfunctions and microbiota composition disorders, which contributes to the inflammatory process and allows their use as metabolic markers of inflammation. This circumstance is confirmed by the results of scientific studies, which proved that succinic acid is a pro-inflammatory signalling molecule that induces interleukin-1β via transcription factor 1α induced by hypoxia. In addition, we revealed significant negative correlations between the levels of representatives of probiotic bacteria in the feces (Bifidobacterium spp. and Faecalibacterium prausnitzii) and the concentration of succinic acid in the blood serum (rs=–0.343 [p=0.026] and rs=–0.430 [p=0.005] respectively).

The major part of circulating fatty (monocarboxylic) acids consists of palmitic, stearic, oleic, linoleic and arachidonic acids. Linoleic acid is the only essential fatty acid whose biological role is due to the participation in the synthesis of arachidonic acid and in the formation of phospholipids of cell membranes. A change in the concentration of monocarboxylic acids is observed not only in metabolic disorders, but also in diseases caused by chronic inflammation [20]. According to our data, an independent change in the concentration of monocarboxylic acids did not have statistically significant deviations in comparison with the parameters of the control group, which does not allow using them as specific markers of celiac disease. In this connection, the ratio AA to EDA was calculated, which determines the biotransformation of linoleic acid, as well as the balance between the pro-inflammatory and anti-inflammatory components of the pool of ω-6-PUFA, linoleic acid derivatives. The results of the study prove the possibility of using this index to assess the activity of chronic inflammation in the intestine.

The course of inflammatory process in the intestine with celiac disease is characterized by multifactorial etiology and

the development of significant metabolic disorders, with the involvement of other metabolically active organs and tissues (liver, mesenteric adipose tissue, central nervous system). An increased B. fragilis/F. prausnitzii ratio can serve as available biomarker for intestinal pro-inflammatory dysbiosis in CD. Altered metabolic pathways and individual metabolites, both endogenous and microbial, involved in these processes (succinate, fumarate, lactate, 2-hydroxybutyric, 2-hydroxyisovalerianic and other hydroxy acids, some phenylcarboxylic and indolecarboxylic acids) can serve as potential diagnostic tools (biomarkers) and/or therapeutic targets for celiac disease. Significant changes in serum levels of microbial and endogenous metabolites, reflecting some metabolic pathways disturbances (glycolysis, TCA cycle, fatty acid metabolism, ketone body metabolism, phenylalanine, tyrosine and tryptophan metabolism, microbial metabolism) are observed in CD. Some metabolites (e. g., a microbial metabolite 2-HIVA), as well as a new metabolomic index (AA/EDA ratio), reflecting the balance between pro-inflammatory and anti-inflammatory components of the omega-6 fatty acid pool, may be considered as candidate biomarkers of chronic intestinal inflammation and metabolic dysbiosis in CD.

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