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ОСОБЕННОСТИ СУБПОПУЛЯЦИОННОГО СОСТАВА РЕГУЛЯТОРНЫХ Т-ЛИМФОЦИТОВ И МИКРОБИОТЫ КИШЕЧНИКА ПРИ СИНДРОМЕ РАЗДРАЖЕННОГО КИШЕЧНИКА

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Резюме. При помощи метагеномного анализа (16 S rRNA) выявлены особенности состава кишечного микробиоценоза у больных синдромом раздраженного кишечника (СРК): 1) увеличение представительства *Actinobacteria*, в том числе *Bifidobacterium* spp., *Firmicutes*, в том числе принадлежащих к семействам *Streptococcaceae* (*Streptococcus*), *Lachnosperaceae* (*Dorea*), *Veillonellaceae* (*Dialister*), *Proteobacteria* (семейства *Enterobacteriaceae и Desulfovibrionaceae*); 2) уменьшение популяции *Bacteroidetes*, в том числе представителей семейств *Prevotellacea* (*Prevotella* spp.), *Bacteroidaceae* (*Bacteroides* spp.), фирмикутных бактерий, относящихся к семействам *Clostridiaceae* и *Ruminococcaceae* (*Fecalibacterium* spp.). Проточная цитометрия при исследовании субпопуляционного состава Т-регуляторных (Treg) лимфоцитов выявила у больных СРК увеличение количества $CD45R0^+CD62L^+$ клеток центральной памяти (CM), способных регулировать процессы созревания и дифференцировки лимфоцитов в лимфоидной ткани. Обнаружено снижение экспрессии экзонуклеаз CD39 и CD73, что может оказывать существенное влияние на их активность. Отмечено снижение эффекторных клеток памяти (EM) Treg.

Изменения в уровне экспрессии экзонуклеаз CD39 и CD73 находились в обратной корреляционной связи с содержанием протеобактерий и представительством родов *Bifidobactrium* и *Faecalibacterium*. Количественное содержание CM Treg находилось в прямой корреляционной связи с содержанием *Dorea* spp.

Полученные результаты могут указывать на нарушения в процессах дифференцировки Treg, которые тесно связаны с изменениями ключевых компонентов кишечного микробиоценоза при СРК.

Ключевые слова: кишечный микробиоценоз, метагеномный анализ, проточная цитометрия субпопуляции Т-лимфоцитов

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PROFILE OF SUBPOPULATION COMPOSITION OF REGULATORY T LYMPHOCYTES AND INTESTINAL MICROBIOTA IN PATIENTS WITH IRRITABLE BOWEL SYNDROME

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Abstract. The following specificcharacteristics of the composition of intestinal microbiota in patients with irritable bowel syndrome (IBS) were identified using a metagenomic analysis (16 S rRNA): 1) an increase in the representation of *Actinobacteria*, including *Bifidobacterium* spp., *Firmicutes,* including representatives of *Streptococcaceae* (*Streptococcus*), *Lachnosperaceae* (*Dorea*), *Veillonellaceae* (*Dialister*), *Proteobacteria* (*Enterobacteriaceae* and *Desulfovibrionaceae* families); 2) a decrease in the population of *Bacteroidetes*, including representatives of the families *Prevotellacea* (*Prevotella* spp.), *Bacteroidaceae* (*Bacteroides* spp.). *Firmicutes* belonging to the families *Clostridiaceae* and *Ruminococcaceae* (*Fecalibacterium* spp.).

Flow cytometry in the study of the subpopulation composition of T regulatory (Treg) lymphocytes in patients with IBS revealed an increase in the number of $CD45R0^+CD62L^+$ central memory cells (CM), which can regulate the processes of maturation and differentiation of lymphocytes in lymphoid tissue. A decrease in the expression of exonucleases CD39 and CD73 was detected, which can have a significant effect on their activity. A reduction in effector memory cells (EM) Treg was observed.

Changes in the expression level of exonucleases CD39 and CD73 were inversely correlated with the content of *Proteobacteria* and the representation of the genera *Bifidobacterium* spp. and *Faecalibacterium* spp. The content of CM Treg was directly correlated with the content of *Dorea* spp.

The results may be indicative of impairment in the processes of Treg differentiation, which are closely related to changes in key components of intestinal microbiocenosis in IBS.

Keywords: intestinal microbiocenosis, metagenomic analysis, flow cytometry of a subpopulation of T lymphocytes

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Introduction

Irritable bowel syndrome (IBS) is one of the most common functional diseases of the gastrointestinal tract, which affects more than 40% of gastroenterology patients. Significant heterogeneity of the disease is a predictor of the low effectiveness of treatments prescribed to these patients, which in turn leads to the progression of symptoms, a decrease in quality of life, and to an increase in time on sick leave [1, 5, 13, 19, 34].

Studies in recent years have revealed the multifactor nature of the pathogenesis IBS, the presence of disorders of neurohumoral mechanisms of the gastrointestinal tract and the whole body. The cellular composition and morphofunctional characteristics of the intestinal mucosa in IBS have been thoroughly studied. This disease, a condition in the gastrointestinal tract, is more often interpreted not as dysbiosis, but as "low-grade inflammation" [4, 37]. The microbiota of IBS patients demonstrated an increase in the representation of phylum *Proteobacteria*, changes in the ratio of individual representatives of phylum *Bacteroidetes* and *Firmicutes*, a decrease in biodiversity [25, 26, 36]. Most often, IBS increased the population of enterobacteria [36], lipopolysaccharides (LPS) of the cell wall, which can have a direct effect on immunocompetent cells, interacting with TLR4 receptors on their surface [20, 29].

Also, unsaturated fatty acids (butyric, propionic and acetic), which are produced by other representatives of the intestinal microbiocenosis, including those of the *Bacteroidetes* and *Firmicutes* phyla, play an important role in regulating the immune response in patients with IBS [14, 21, 32, 33]. All of the above factors affect the polarization of the immune response.

In IBS, significant changes in the composition of immunocompetent cells at the local and system levels have been identified [3, 7, 17]. The content of mast cells, intraepithelial lymphocytes and enterochromaffin cells (endocrine cells producing 80% of all serotonin synthesized in the human body) is elevated in the cellular infilatrate in the intestine of IBS patients. Locally and in the peripheral blood of patients, there has been an increase in the number of inflammatory markers: C-reactive protein [11], proinflammatory cytokines – IL-1, IL-6 and TNFα, while the amount of regulatory and anti-inflammatory cytokine IL-10 is generally reduced [3]. These changes in the state of the mucosa, the composition of the microbiota, cytokine status in IBS are accompanied by activation of T lymphocytes [17] circulating in the blood of patients.

Previously, we have shown [18], that the relative content of follicular T helpers (Tfh) with the phenotypes CXCR3-CCR6-CCR4 decreased in IBS patients, while the level of Tfh populations with the properties of Th17 (DN Tfh17 and Tfh17.1) demonstrated a statistically significant increase. These changes in Tfh composition suggest there are changes in how the specific humoral immune response in IBS patients functions, which was supported by reports of an increase in the population of B-lymphocytes previously detected in IBS by other authors [7].

Despite the fact that the exact causes of development and pathogenesis of IBS remain unknown, the involvement of Treg in this pathological process is of interest, as it is the pool of Treg cells that is able to act as regulators of inflammation and as suppressors in the development of allergic and autoimmune reactions of the body.

The main objective of this study was to analyze the subpopulation composition of Treg lymphocytes in peripheral blood in patients with IBS, taking into account the analysis of the gut microbiome.

Materials and methods

Patient characteristics

Twenty-two patients fulfilling the Rome Criteria V for irritable bowel syndrome with diarrhea (IBS-D were selected for this clinical trial. The subjects included 8 men (32%) and 17 women (68%). The mean age in the group was 37.0±8.05 (24-52) years. Patients were monitored in the North-Western State I. Mechnikov Medical University. All patients have signed an informed consent.

The control group was made up of 30 healthy volunteers of similar age and gender, and same proportion of male/ female subjects as in the group of subjects with IBS.

The object of the study was blood samples obtained by the peripheral vein puncture and collected in vacuum tubes with the addition of K_3EDTA. All studies were performed with the informed consent of the subjects and in accordance with the Helsinki Declaration of the World Medical Association "Ethical Principles for Medical Research Involving Human Subjects" with amendments from 2000 and "Rules of Clinical Practice in the Russian Federation" approved by the Order of the Ministry of Health of the Russian Federation 19.06.2003 No. 266. All laboratory tests were carried out on the day of the blood collection.

Study of the composition of the gut microbiota

The composition of intestinal lumen microbiota was identified in the samples of faeces frozen on the day of the material's collection. Metagenomic analysis was used for the analysis. Libraries of hypervariable regions V3 and V4 of the 16S RNA gene were analyzed on MiSeq (Illumina, USA). DNA were isolated from faeces using the kit DNK-EKSPRESS by Litekh (Moscow, Russia). The standard method recommended by Illumina based employing two rounds of PCR was used to prepare the libraries. The first round was the amplification of a 16S RNA gene fragment, adding to the target fragment of the adapter nucleotide sequences included in the PCR primers (Table 1).

The second round of PCR, sample preparation, sequencing and bioinformatic processing was performed at the Resource Center "Biobank" of the St. Petersburg State University. The quality of raw DNA readings was assessed by Fastqc (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The selection of operatiionsl taxonomic units (OTEs) based on similar sequences of 16S rRNA was carried out by the CD-HIT-OTU-Miseq software. CD-HIT-OTU-Miseq allows to select OTE by the sequence of terminal regions without the need to merge paired sequences. Clustering was carried out by comparing the DNA regions R1 and R2 with good reading quality of 200 and 180 nucleotide pairs, respectively, 97% of the homology of the selected sites and cut-

TABLE 1. SEQUENCE OF PRIMERS FOR ANALYSIS OF THE SEQUENCES OF 16S rRNA GENES

Sequence of the primers of V3 and V4 regions of 16S rRNA used		Amplicon size
Forward (341)	tcgtcggcagcgtcagatgtgtataagagacagcctacgggnggcwgcag	464
Reverse (785)	gtctcgtgggctcggagatgtgtataagagacaggactachvgggtatctaatcc	

offs 0.00001 by quantity. The OTE annotation was implemented using Greengenes database version 13.5. OTE presented in less than 5% of samples were dismissed as noise.

Study of the phenotypes of immune cells

Phenotypes of immune cells were determined by flow cytofluorimetry in venous blood obtained by puncture of a peripheral vein and collected in vacuum tubes with the addition of K_3EDTA. Blood samples were taken on the same day as the feces. The preparation of peripheral blood samples and the adjustment of the flow cytofluorimeter was carried out in accordance with the recommendations set forth by S.V. Khaidukov et al. [15]. The following panel of monoclonal antibodies conjugated with various fluorochromes was used to identify the main populations of regulatory T lymphocytes and assess their expression level of CD39 and CD73: CD39-FITC (clone A1, cat. 328206, BioLegend, Inc., USA), CD25-PE (clone B1.49.9, cat. A07774, Beckman Coulter), USACD62L-ECD (clone DREG56, cat. IM2713U, Beckman Coulter), USACD45R0-PC5.5 (clone UCHL1, cat. IM2712U, BeckmanCoulter), USACD4-PC7 (clone SFCI12T4D11 (T4), cat 737660, Beckman Coulter), USACD8-APC (clone B9.11, cat. IM2469, Beckman Coulter), USACD3-APC-Alexa Fluor 750 (clone UCHT1, cat. A94680), CD73-Pacific Blue (clone AD2, cat. 344012, BioLegend, Inc.) and CD45-Krome Orange (clone J33, cat. ABeckman Coulter, USAUSA96416, Beckman Coulter, USA). This set of monoclonal antibodies was used to stain 100 μl of peripheral blood in accordance with the manufacturer's recommendations. To identify the main Treg populations, as well as to analyze their expression of CD39 and CD73, an algorithm (gating strategy) was used, which was described in detail earlier [24].

Removal of red blood cells from samples was carried out following a no-wash procedure using a lysing solution Versa Lyse (cat. No. No A09777), to 975 ml of which 25 ml of IOTest 3 Fixative Solution was added *ex tempera* (Cat. No A07800). After the destruction of red blood cells, the samples were once washed off with an excess of saline solution at 330g for 7 minutes, after which the supernatant was removed, and the cell sediment was resuspended in a saline solution with a pH of 7.2-7.4 containing 2% paraformaldehyde (Sigma-Aldrich, USA). The samples were analyzed on the Navios™ cytofluorimeter (Beckman Coulter, USA), equipped with three 405, 488 and 638 nm diode lasers.

Statistical analysis

Comparative analysis was conducted using ANOVA with post hoc HSD test for unequal sample sizes in Program Statistica 8. Proportions were compared using the Yates correction. Spearman's correlation was used to define statistical relationships between the test paramaters. Differences of $p < 0.05$ were considered significantreliable in all statistical analyses.

Results

Study of the features of the composition of the intestinal microbiocenosis

The analysis of the composition of the gastrointestinal microbiome in patients with IBS was carried out by comparing it with the microbiome of healthy volunteers with similar gender and age characteristics.

Metagenomic analysis showed that at the filum level there is an increase in the representation of *Actinobacteria, Firmacutes* and *Proteobacteria*. At the same time, a decrease in the *Bacteroidetes* population was noted (Figure 1).

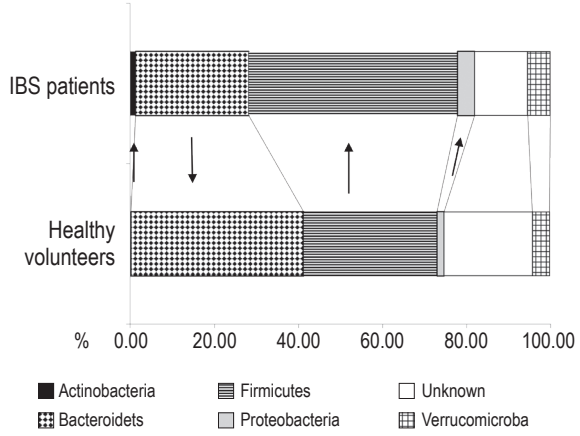

Figure 1. Features of the composition of the gut microbiota in IBS patiens at the phylum level

Note. The arrows indicate statistically significant differences in the representation of individual phyla . ↓ p, decrease; ↑, an increase compared to the group of healthy volunteers. The picture does not show values less than 1%.

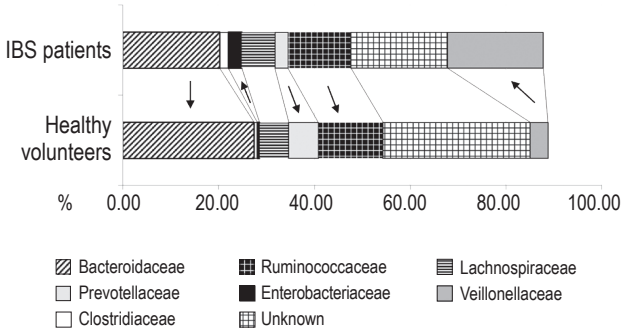

Figure 2. Features of the composition of the intestinal microbiota in IBS at the family level

Note. The arrows indicate statistically significant differences in the representation of individual families. ↓, decrease; ↑, increase in comparison with the healthy volunteers group. The figure does not show values less than 1%.

An analysis of the representation of bacterial families present in the gut mircobiota of healthy volunteers and IBS patients allowed to clarify the taxonomic features of the composition of the population. Thus, the phylum *Bateroidetes* in healthy people was distinguished by a high content of *Bacteroides* and *Proteobacteria*. The representation of the families *Enterobacteriaceae*, *Streptococcaceae* and *Veilonellaceae* in the intestine of patients with IBS was increased, together with a reduction in *Clostridiaceae, Prevotellaceae, Ruminococcaceae* and *Bacteroidaceae* (Figure 2, Table 2).

Analysis of the intestinal microbiome at the level of individual genera of bacteria revealed: a decrease in the representation of *Bacteroidetes* and *Faecalibacterium* spp. against the background of an increase in representatives of the genera *Dorea, Bifidobacterium*, *Desulfovibrio, Streptococcus* and *Dialister* (Table 2).

Analysis of subpopulation composition of peripheral blood T lymphocytes

Content of major populations of T lymphocytes of peripheral blood of IBS patients

The study of cluster differentiation of the main populations of T lymphocytes showed that peripheral blood samples obtained from IBS patients and healthy volunteers are not statistically different in terms of the absolute content of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells (data not shown). The relative content of regulatory T cells with the phenotype $CD4^+CD25^{bright}$ also did notstatistically differ between the groups (63 cells/μl (42-84) in patients with IBS and 57 cells/μl (44-73) in healthy volunteers (p = 0.658). The relative cell content of this population did not change within the general population of peripheral blood lymphocytes (3.09% (2.62-3.74) *vs* 3.38% (2.71-3.88) at the p = 0.213), and when analyzing the relative content of Tregs among $CD3^+CD4^+$ T helpers 6.48% (5.86-7.80) *vs* 6.78% (6.05-8,34) at p = 0.335.

Analysis of subpopulation composition of regulatory T lymphocytes of peripheral blood of IBS patients by level of expression of CD45R0 and CD62L

In order to detect individual peripheral blood Treg subpopulations, the level of expression on the surface of cells of two antigens was evaluated: CD45R0 (short form of antigen CD45, indicating differentiation of T lymphocytes into central or effector memory cells) and CD62L (adhesion molecule allowing T lymphocytes to enter peripheral lymphoid organs through high endothelial venules) [24]. Based on this analysis three main populations of Treg were identified: naive or thymic cells with $CD45R0^-CD62L^+$ phenotype and two populations of peripheral Treg – central and effector memory cells with $CD45R0^+CD62L^+$ and $CD45R0^+CD62L^-$ phenotypes, respectively. The analysis of the relative and absolute content of these cell populations is shown in Figure 3. Thus, there was

TABLE 2. REPRESENTATION OF STATISTICALLY SIGNIFICANT TAXA IN THE FAECES OF IBS PATIENTS AND HEALTHY VOLUNTEERS IN %

Taxons	Name of the taxons	Healthy volunteers	Patients with IBS	Changes as compared to the volunteers
Phylum	*Actinobacteria*	0.13	1.24	↑
	Firmacutes	31.71	49.69	↑
	Proteobacteria	1.68	4.02	↑
	Bacteroidetes	41.09	26.92	↓
Family	*Enterobacteriaceae*	0.43	2.84	↑
	Veilonellaceae	3.74	20.0	↑
	Clostridiaceae	0.67	1.72	↓
	Prevotellaceae	6.19	2.71	↓
	Ruminococcaceae	13.52	13.16	↓
	Bacteroidaceae	27.44	20.24	↓
	Streptococcaceae	0.15	0.64	↑
Genus	*Fecalibacterium*	2.5	0.8	↓
	Bacteroides	39.1	21.2	↓
	Dorea	0.00085	0.00211	↑
	Bifidobacterium	0.00086	0.01475	↑
	Desulfovibrio	0.000291	0.002832	↑
	Streptococcus	0.00291	0.017398	↑
	Dialister	0.079716	0.11864	↑

a significant increase (p = 0.026) in the proportion of Treg with phenotype $CD45R0^+CD62L^+$ in IBS patients from 57.45% (49.57-60.15) to 63.24% (55.96-66.76), which was accompanied by a decrease in the level of $CD45R0^+CD62L^-$ Treg in circulation (p < 0.001), from 11.34% (9.15-14.77) to 6.88% (4.24-8.44).

Analysis of the expression of effector molecules CD39 and CD73 by regulatory T lymphocytes of peripheral blood of IBS patients

As a part of further studies of Treg phenotype in IBS patients the expression of two membrane-associated enzymes was analyzed: CD39 (or E-NTP Dase1, Ectonucleoside triphosphate diphosphohydrolase-1) and CD73 (or Ecto5'NTase, ecto-5'-nucleotidase) with nucleotidase activity. It has been shown that in patients with IBS and in healthy volunteers there is not statistically significant difference in relative content of CD39 Treg (30.21% (8.49-47.02) and 40.10% (30.41-50.00), respectively, at p = 0.228), respectively, and of CD73 Treg (4.46% (2.66-5.47) and 4.81% (3.53-6.01), respectively, at p = 0.220).

However, in analyzing the expression of these surface molecules on various subpopulations of Treg of identified on the basis of CD45R0 and CD62L coexpression, significant differences were found between the groups compared, which concerned naive or thymic $CD45R0^-CD62L^+$Treg (Figure 4). Thus, an almost twofold reduction in the content of $CD39^+$ naive Treg and CD73-positive cells of this population in patients with IBS has been demonstrated: from 14.36% (6.34-18.78) to 6.25% (1.68-9.28, p < 0,001) and from 5.44% (3.93-7.18) to 2.87% (0.88-3.98, p < 0,001), respectively.

The summarized results obtained in the study of cluster differentiation of Treg lymphocytes are presented in the Table 3.

Correlation between the indicators of cluster differentiation of lymphocytes and the representation of individual taxons of bacteria in IBS patients

Spearman's correlation test used to analyze data for the the group of patients with IBS revealed a direct correlation (r = 0.483744, p < 0.05) between representatives of the *Dorea* spp. and $CD45R0^+CD62L^+$Treglymphocytesofcentralmemory (Figure 5).

A negative correlation was established between the representation of phylum *Proteobacteria*, genera *Faecalibacterium* and *Bifidobacterium*, and Treg lymphocytes of central memory, on which two or one of the following enzymes was expressed: diphosphohydrolase and nucleotidase ($CD73^+$ and/or $CD39^+$ molecules) (Figures 6-8).

Discussion

IBS is one of the most common human gastrointestinal diseases, at the same time it is difficult to diagnose since the intestinal pathology is closely associated with psycho-somatic disorders. For that reason it is extremely important to discover characteristic pathogenesis mechanisms and specific properties of innate immune cell differentiation. This work was used to trace the correlation between the expression of marker molecules on the surface of immunocompetent cells and changes in the composition of microbiocenosis. It is no secret that the microbiota of patients with IBS is subject to significant disbiotic changes, however, the available in the literature data on the structure of microbiota in patients with IBS are quite contradictory.

The increase of the *Proteobacteria* quantity, including the *Enterobacteriaceae* family, has previously been noted by many authors, [25, 26, 27, 28, 36]. This study showed that the growth of enterobacteriae population is accompanied by an increase in the content of *Deltaproteobacteria, – Desulfovibrio*, capable of partially neutralizing toxic and radioactive compounds as well as of causing superficial destruction of other bacteria [16, 35], as well as cause superficial destruction of other bacteria. In addition to the usual controlled shifts, an increase of bacteria belonging to the genera *Weilonella*, *Lahnospiria, Streptococcus, Bifidobacteria* and decrease of *Bacteroides spp* is present. Numerous studies of gut microbiota in IBS have noted an increase in some types of microorganisms with "pro-inflammatory" activity [24, 26], including representatives of the *Dorea*, *Ruminococcus* and *Clostridium* genera. It should be noted, that the presence of Dorea (a member of the *Lachnospiraceae* family) was closely associated with an increase in pro-inflammatory cytokine production.

TABLE 3. REPRESENTATION OF STATISTICALLY SIGNIFICANT INDICATORS IN THE EVALUATION OF Treg SUBPOPULATIONS IN THE PERIPHERAL BLOOD OF PATIENTS WITH IBS AND HEALTHY VOLUNTEERS

Indicator markers	Healthy volunteers	Patients with IBS	Change compared to healthy volunteers
$CD45R0^+CD62L^+$, %	57.45*	63.24	↑
$CD45R0^+CD62L^-$, %	11.34	6.88	↓
$CD45R0^-CD62L^+CD39^+$, %	14.36	6.25	↓
$CD45R0^-CD62L^+CD73^+$, %	5.44	2.78	↓

Note. *, % from total amount of Treg.

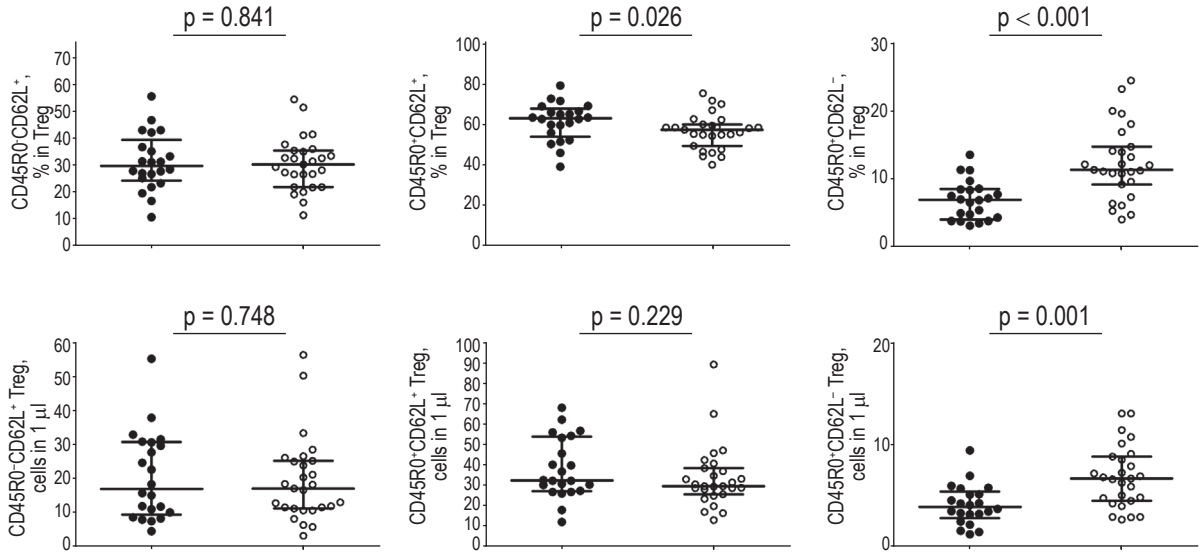

Figure 3. Changes in the subpopulation composition of peripheral blood regulatory T cells

Note. The upper and lower rows of histograms (from left to right) are the relative and absolute contents of regulatory peripheral blood T lymphocytes with phenotypes $CD45R0^-CD62L^+$, $CD45R0^+CD62L^+$ and $CD45R0^+CD62L^-$, respectively. Black, patients with IBS (n = 25); white, healthy volunteers (n = 30). The results are presented as median and interquantile range (Me ($Q_{0.25}$-$Q_{0.75}$). The nonparametric Mann–Whitney test was used to compare the samples.

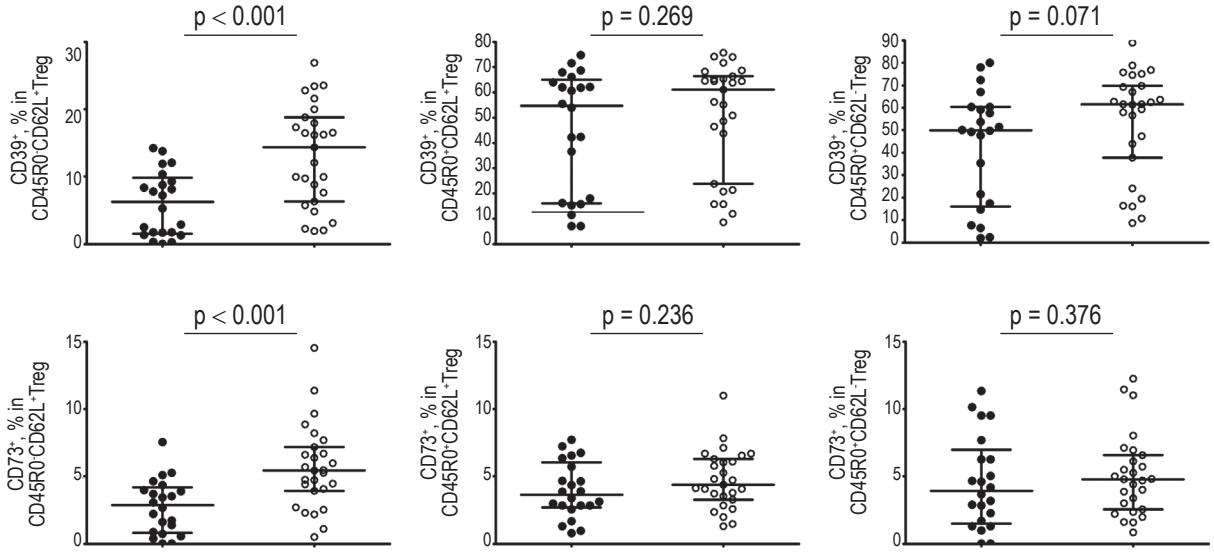

Figure 4. Expression of CD39 and CD73 by various subpopulations of regulatory peripheral blood T cells in IBS patients

Note. The upper row (from left to right) is the content of $CD39^+$ cells relative to $CD45R0^-CD62L^+$, $CD45R0^+CD62L^+$ and $CD45R0^+CD62L^-$Treg, respectively. The bottom row of histograms (from left to right) is the content of $CD73^+$ cells relative to $CD45R0^-CD62L^+$, $CD45R0^+CD62L^+$ and $CD45R0^+CD62L^-$Treg, respectively. Black, patients with IBS (n = 25); white, healthy volunteers (n = 30). The results are presented as median and interquantile range (Me ($Q_{0.25}$-$Q_{0.75}$). The nonparametric Mann–Whitney test was used to compare the samples.

The ability of these bacteria to metabolize sialic acids could be accompanied by increase of permeability and further development of inflammatory reactions in the intestine [31]. The ability of this kind of bacteria to form a significant compared to other representatives of the gut microbiota quantity of hydrogen and carbon dioxide largely explains the symptoms of bloating and pain in the intestines characteristic of IBS [38].

It has been shown that the increase in streptococcus content led to stimulation of the production of pro-inflammatory cytokine IL-6 and the degradation of mucin [26], so the increase in the representation of

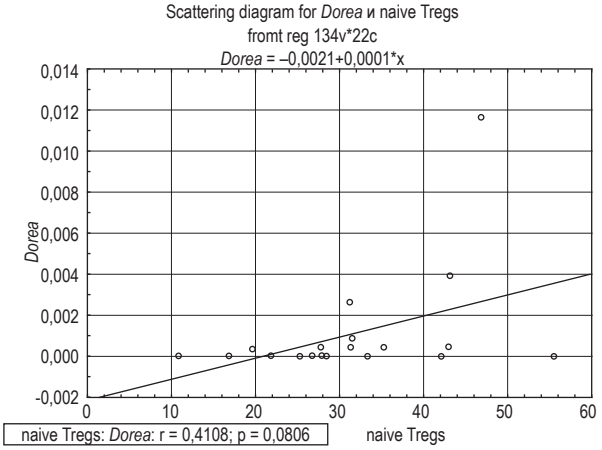

Figure 5. Direct correlation (r = 0.483744, p < 0.05) between the content of naive Tregs (% of Treg) and the representation of *Dorea* spp. (%)

Note. Naive or thymic cells ($CD45RA^+CD62L^+$% relative to the total number of naive T helpers with phenotype $CD45RA^+CD62L^+$.

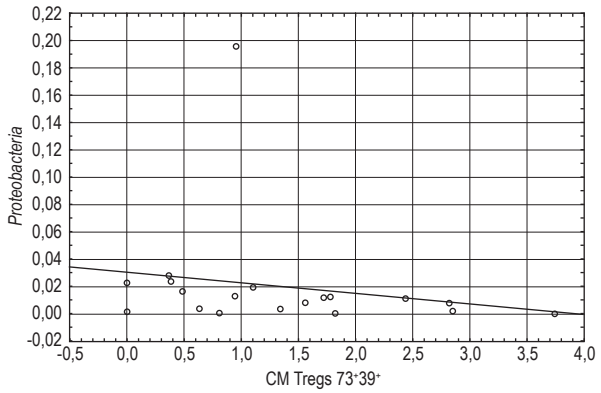

Figure 6. Correlation (r = -0.459851, p < 0.05) between the cluster differentiation indices of $CD45RA\text{-}62L^+CD39^+CD73^+$ lymphocytes (% relative to the total number of T helper cells) and *Proteobacteria* representation (%) in patients with IBS

these bacteria in IBS patients, identified in this work, was predictable.

As in other reports on the composition of intestinal microbiota of patients with IBS a decrease in beneficial resident *F. prausnitzii* bacteria producing butyrate has been found in this work. Many authors associate the increase in these bacteria with a favorable prognosis for the progression and course of non-specific ulcerative colitis and Crohn's disease [2]. There is a correlation between the presence of *F. prausnitzii* (together with *Butyricococcus pullicaecorum* and other butyrate-producing bacteria) in the mammalian gastrointestinal tract and a decrease in TNFα and IL-8 levels, as well as an increase in production of IL-10 [6, 9, 22].

The excessive increase in bifidobacteria, as well as the increase in the representation of *Actinobacteria* was unexpected. However, these changes can be associated with the launch of a compensatory reaction of the organism to the formation of a low-grade inflammation site in the intestine of patients with IBS. Further studies may involve an analysis of species and strains of *Bifidobacterium* spp.

Treg play a leading role in regulating the functional activity of immune system cells in both peripheral lymphoid and in inflamed tissues [10]. The analysis of the relative and absolute content of Treg in the peripheral blood of IBS patients did not demonstrate any differences in these indicators when compared with the control group, which is fully consistent with the results given in the literary sources [12].

The analysis of our results, based on the study of the subpopulation composition of Treg, has identified a statistically significant increase in the population of $CD45R0^+CD62L^+$CM cells, which can regulate the maturation and differentiation of lymphocytes in lymphoid tissue, including lymph nodes and Peyer's patches.

At the same time, we have noted a significant decrease in both the relative and absolute content of EM Tregs, the main function of which is believed to be regulating immune reactions occurring in peripheral tissues, including connective tissue of the intrstibal wall. A drop in the circulation of these cells may be related both to an increase of their migration to inflamed tissues and to abnormalities in the mechanisms of their differentiation in lymphoid organs related to a decrease in mature and Tregs capable of systematic migration to the periphery.

Moreover, sources indicate that the expression of homing molecules on $CD4^+$ lymphocytes in IBS increases, which are associated with an increase in CD levels of 62L and z4-7-integrin [23]. However, the changes described by the authors also seem to apply to Treg, as we have shown in the course of the study.

One of the most important mechanisms of suppression of inflammation, which is realized by Treg both in lymphoid tissue and in the site of inflammation, is the degradation of pro-inflammatory extracellular ATP to adenosine, which has a wide spectrum of anti-inflammatory action and acts on the effector cells of innate and acquired immunity [8]. When comparing $CD39^+$ and $CD39^-$Treg *in vitro*, it was shown that the presence of CD39 is closely related to the high ability of these cells to show their suppressor properties due to the efficient expression of the transcription factor FoxP3, as well as the regulatory molecules CTLA4, GARP, and LAP [28].

In the course of our research we found a decrease in the density of CD39 and CD73 expression on the surface of "naive" Treg, which can significantly affect the functions performed by these cells. It

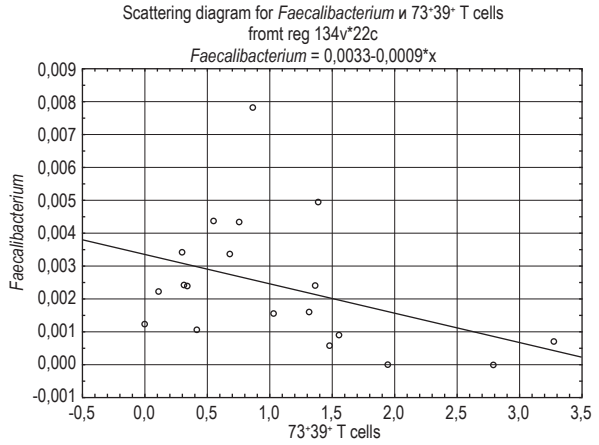

Figure 7. Correlation (r = -0.460728, p < 0.05) between the indicators of cluster differentiation of lymphocytes CD39+CD73+ of T cells and representation of the genus *Faecalibacterium* in patients with IBS

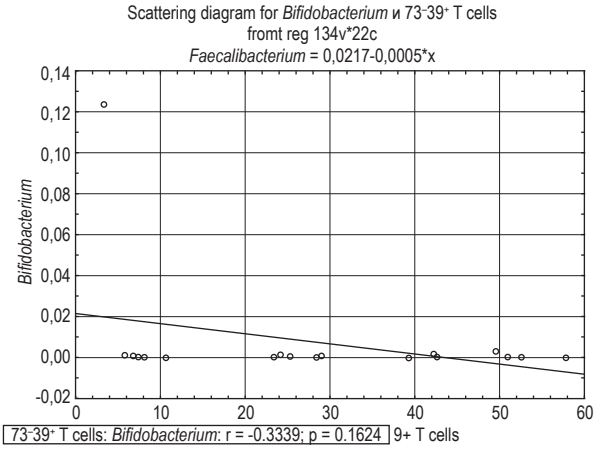

Figure 8. Correlation (r = -0.456460, p < 0.05) between the indicators of cluster differentiation of lymphocytes CD39+CD73+ of T cells and the representation of *Bifidobacterium* (%) in patients with IBS

should be especially noted that CD45R0-Treg, which have passed only the stage of antigen-independent differentiation in the thymus, is capable of recognizing only the body's own antigens and not the antigens of exogenous origin [30]. Apparently, this very population of cells plays a leading role in maintaining tolerance to autoantigens and prevents the development of autoimmune processes in the body. The results obtained may indicate abnormalities in the processes of Treg differentiation, which may lead to the disruption of peripheral tolerance in IBS.

The composition of the intestinal microbiota and the characteristics of cluster differentiation of subpopulations of T lymphocytes were evaluated in patients of IBS simultaneously. Therefore, the negative correlations between the decrease in CM lymphocytes functioning as suppressors of the inflammatory response and the increase in proteobacteria and the maingas producers of the genus *Dorea* spp. seems very significant and important.

On the other hand, the identified negative correlations between regulatory cell populations and bifidobacteria and fecal bacteria, which are usually correlated with positive changes in the state of intestinal microbiota are difficult to explain. It is possible that in the latter case there is a compensatory reaction of the intestinal microbiota to the chronic inflammation developing in the gut.

The discovered tendency to trigger inflammatory reactions, noted earlier [18], and the discovered abnormalities in systematic and local regulation of immunological processes associated with intestinal dysbiosis create the preconditions for the possible development of an autoimmune pathology associated with an increase in the number of naive thymic lymphocytes and possible deficiencies of immunological tolerance to their own antigens and antigens to representatives of resident microbiota.

The results of the study suggest that changes in the level of individual microorganisms in the microbiota and the subpopulation composition of Treg cells are related, and the correction of the abnormal composition of microbiota of the intestine may represent a new strategy to change the immune status of patients with IBS. In addition, the identified patterns open up new diagnostic possibilities to determine the degree of progression of dysbiosis in IBS using flow cytometry. On the other hand, the identified new features of the microbiota of patients with IBS can also contribute to a more accurate diagnosis of the disease, opening up additional prospects for controlling its course.

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