ORIGINAL ARTICLE

FEATURES OF INTESTINAL MICROBIOTA IN PATIENTS WITH NONALCOHOLIC FATTY LIVER DISEASE: EFFECTS ON MARKERS OF INFLAMMATION AND HEPATIC STEATOSIS

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ABSTRACT

The aim: To study the state of the intestinal microbiota (IM) in patients with Nonalcoholic fatty liver disease (NAFLD) and to determine changes in its composition at the level of basic phylotypes.

Materials and methods: The study included 114 patients with NAFLD with metabolic disorders and 64 patients of control group. Determination of the composition of the IM at the level of major phylotypes was performed by identifying total bacterial DNA and DNA of *Bacteroidetes, Firmicutes* and *Actinobacteria* by quantitative polymerase chain reaction (PCR) in real time (qRT-PCR) using universal primers for the 16S rRNA gene and taxon-specific primers of production (Thermo Fisher Scientific).

Results: It was defined the weak correlation between the content of *Firmicutes* and proinflammatory markers (C-reactive protein (CRP) and Tumor necrosis factor (TNF) alpha) ($p < 0.05$) and inverse correlation of CRP with the content of *Bacteroidetes* ($p < 0.001$). Also have been observed significant changes in the main intestinal phyla in the direction of increasing the content of *Firmicutes* in patients with NAFLD with a high degree of steatosis and elevated levels of proinflammatory cytokines ($p < 0.05$).

Conclusions: IM imbalance leads to excessive synthesis of pro-inflammatory cytokines, promotes the activation of cellular mechanisms, which increases the flow of fatty acids into hepatocytes and increases the degree of hepatic steatosis.

KEY WORDS: Nonalcoholic fatty liver disease, intestinal microbiota, proinflammatory markers, hepatic steatosis

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of disease that is caused by various mechanisms, including dietary, metabolic, genetic, environmental and microbiotic factors. A number of experimental and clinical studies have demonstrated evidence of a close relationship between NAFLD and intestinal dysbacteriosis [1, 2]. Nowadays, the study of the microbiota and its role in the pathogenesis of NAFLD have become extremely relevant.

Trillions of microbes that colonize the human body, including bacteria, archaea, viruses and eukaryotic microbes, spread along the length of the gastrointestinal tract (GIT) [3].

The combination of a small number of pathogens and a large number of key genera of bacteria characterizes the healthy state of the IM [4]. Factors influencing the state of the microbiota include: genetics, diet, method of childbirth, geographical location, the impact of drug treatment and others [5, 6]. As a result, the IM is unique to each person and at the same time under the influence of various factors changes throughout life. In it's turn, the IM affects the metabolic phenotype of the host, participates in food and drug metabolism and improves the immune system [7, 9-11].

The strategy to demonstrate the causal role of the IM in the pathogenesis of NAFLD is to investigate the association of the whole microbiome and to analyze potential key intestinal microbial phylotypes that may be associated with the etiology or development of a particular chronic disease. Basically, there are six different phylotypes in the IM: *Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Actinobacteria* and *Fusobacteria* [12]. These bacteria may be involved in a variety of important metabolic processes, including regulation of polysaccharide levels, bile acid production, choline metabolism, energy intake, stimulation of endogenous ethanol production, and protection against pathogens [13-15].

The integration of current available data supports the hypothesis that mild, systemic, and chronic inflammation caused by the IM may initiate and exacerbate the development of metabolic diseases such as obesity, diabetes, and NAFLD in humans [16-18].

Despite recent studies on human beings and animals have shown the connection between intestinal dysbacteriosis and NAFLD many questions remain open.

THE AIM

The aim of the stage of our research is to study the state of the IM in patients with NAFLD and associations of the main phylotypes with risk factors for the development and progression of NAFLD.

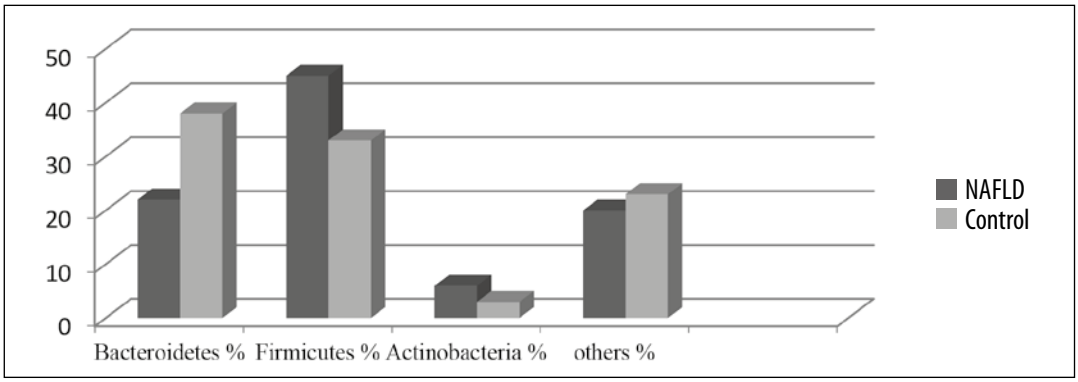

Fig. 1. Relative composition of IM at the level of basic phylotypes in patients with NAFLD and control group.

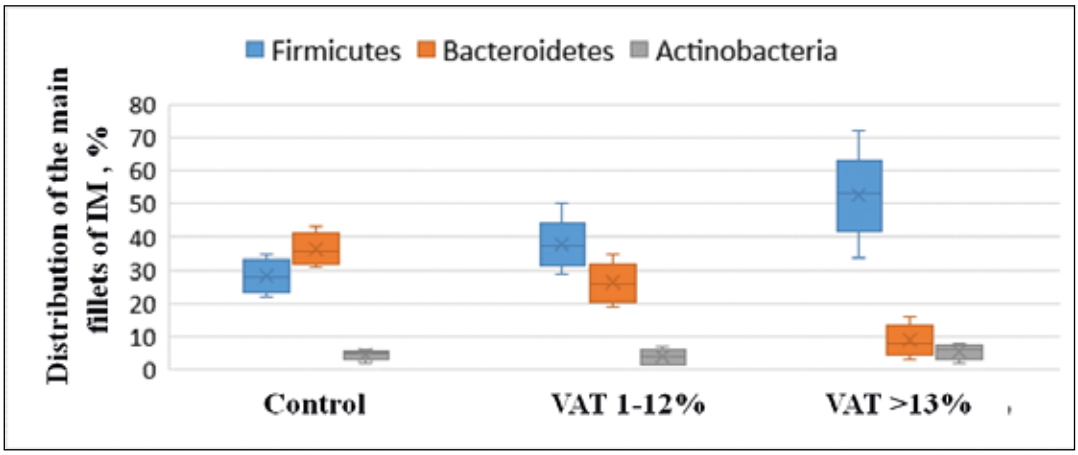

Fig. 2. Distribution of the main fillets of IM in the examined patients with NAFLD depending on the percentage of VAT.

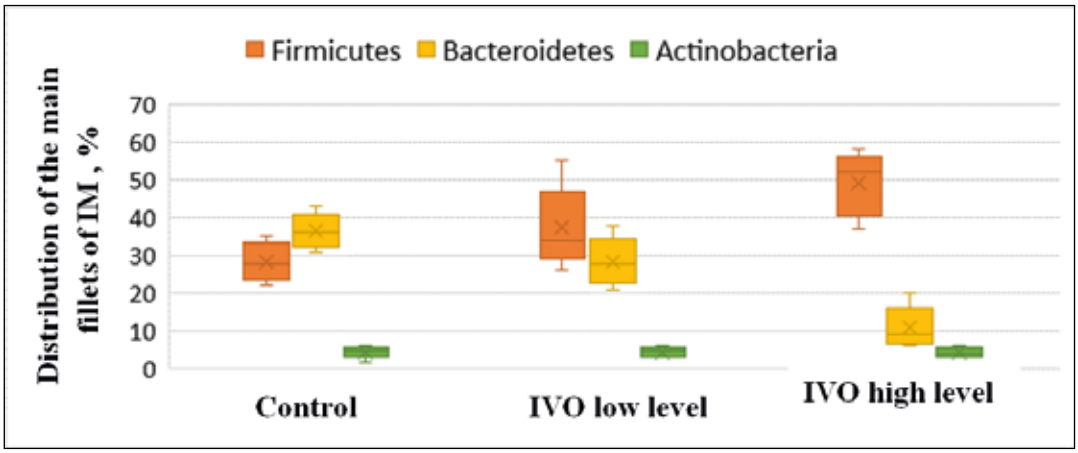

Fig. 3. Changes in the content of IM of patients with NAFLD depending on the activity of VAT.

MATERIALS AND METHODS

The research was conducted in the department of studying diseases of the digestive system and their comorbidity with non-infectious diseases of the Government Institution "L.T. Malaya Therapy National Institute of the National Academy of Medical Sciences of Ukraine" (certified license № AE 197294 dated 06.06.2013, Ministry of Health of Ukraine).

The study included 114 patients with NAFLD with metabolic disorders, 64 patients of control group, who were examined on the basis of the Department of Gastroenterology and Therapy and the outpatient department of the Government Institution "L.T. Malaya Therapy National Institute of the National Academy of Medical Sciences of Ukraine". Gender distribution was reciprocal. The mean age of the examined patients with NAFLD was (52.56 ± 11.7) years.

Estimation of anthropometric parameters included measurement of growth and determination of body weight with calculation of body mass index (BMI). All patients were evaluated for the function of liver, carbohydrate metabolism and lipid metabolism.

To study the body composition of patients (determination of total % of body fat, % visceral adipose tissue (VAT) it was used an electronic device - body weight monitor OMRON BF-511 (Japan, 2011). To determine the dysfunction of VAT the index of visceral obesity was calculated (IVO) by the method of Amato M.C. [1].

Determination of serum C-reactive protein (CRP) levels was performed by enzyme-linked immunosorbent assay

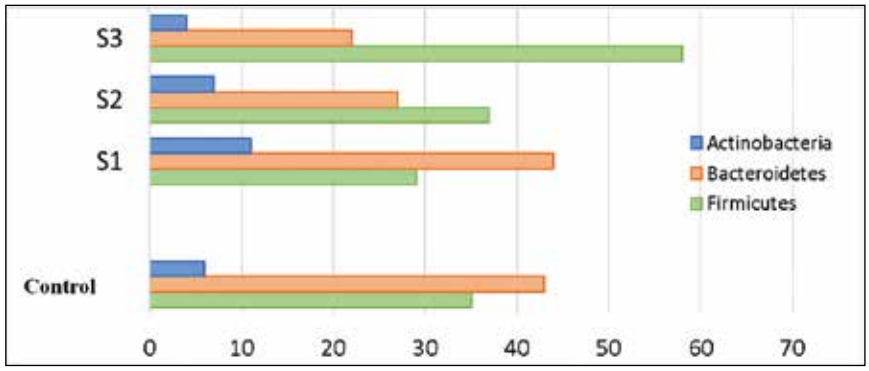

Fig. 4. The content of the main fillets of IM (%) of patients with NAFLD depending on the degree of hepatic steatosis.

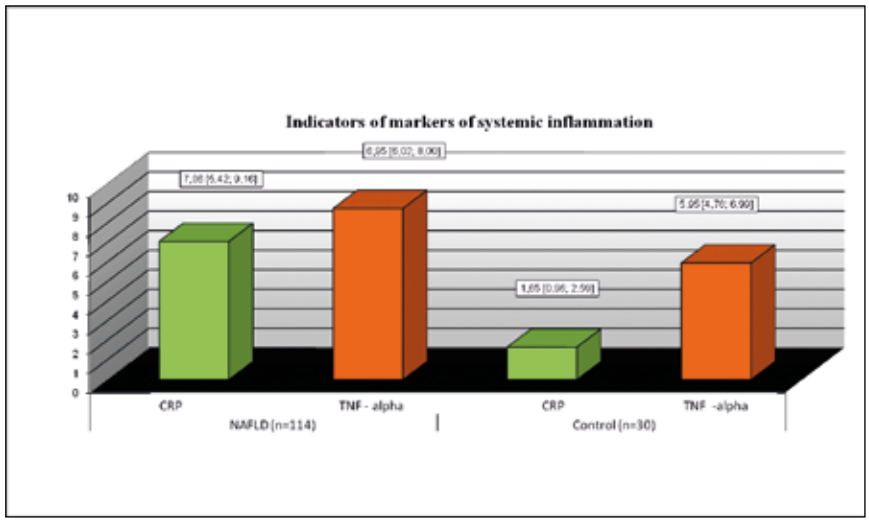

Fig. 5. Indicators of markers of systemic inflammation in patients with NAFLD.

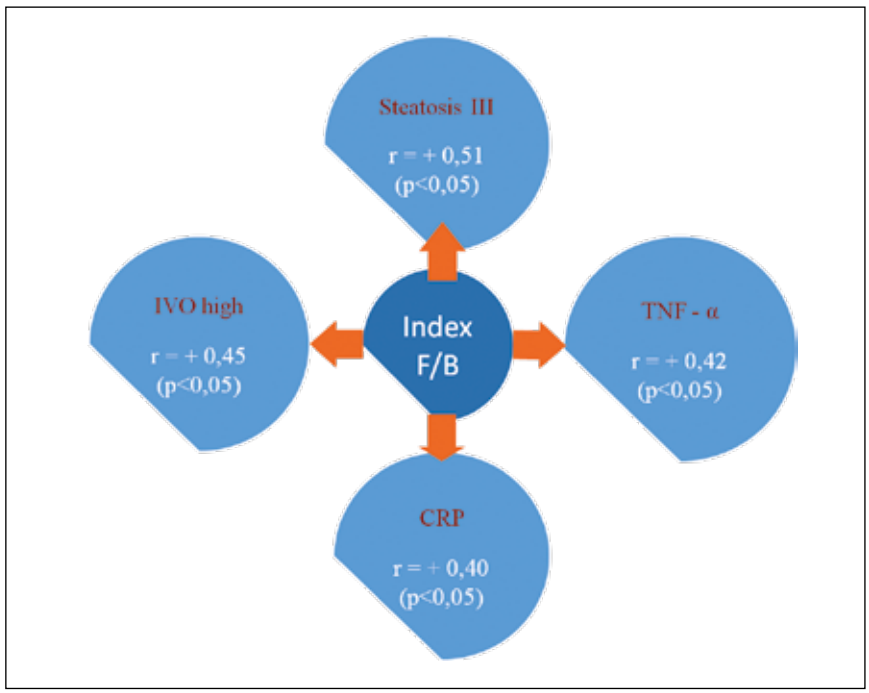

Fig. 6. Dependence of the ratio of major intestinal fillets (Firmicutes / Bacteroidetes) on markers of systemic inflammation (CRP and TNF-alpha), steatosis and activity of visceral obesity (IVO) of patients with NAFLD.

using hs-CRP ELISA KIT reagent kits - DRG International Inc. (USA) according to the manufacturer's instructions.

The level of Tumor necrosis factor (TNF) alpha in the serum was determined by using a set of reagents «ELISA-TNF-alpha», series 154 for the quantification of tumor necrosis factor in serum by the ELISA method.

The degree of steatosis levels was assessed by determining the wave attenuation coefficient (WAC) and performing shear wave elastometry (SWE), respectively.

Determination of the relative composition of the main phylotypes of the IM was performed by the method of molecular genetic research. The DNA concentration in the

Table I. Dependence of the main fillets of IM on markers of systemic inflammation.

	N	Spearman coefficient	t(N-2)	p
CRP & % *Bacteroidetes*	114	-0,298117	-3,66890	0,000347*
CRP & % *Firmicutes*	114	0,0249397	3,02535	0,002963*
CRP & % *Actinobacteria*	114	0,061222	0,72054	0,472409
CRP & % other	114	-0,118557	-1,40262	0,162974
TNF & % *Bacteroidetes*	114	-0,074736	-0,88041	0,380167
TNF & % *Firmicutes*	114	0,178312	2,12880	0,035047*
TNF & % *Actinobacteria*	114	-0,070311	-0,82802	0,409089
TNF & % other	114	-0,086554	-1,02061	0,309224

* - <0.05 relationships are valid

Table II. The dependence of the main fillets of IM on markers of systemic inflammation of patients with overweight and NAFLD.

	N	Spearman coefficient	t(N-2)	p
CRP & % *Bacteroidetes*	114	-0,298117	-3,66890	0,000347*
CRP & % *Firmicutes*	114	0,0249397	3,02535	0,002963*
CRP & % *Actinobacteria*	114	0,061222	0,72054	0,472409
CRP & % other	114	-0,118557	-1,40262	0,162974
TNF & % *Bacteroidetes*	114	-0,074736	-0,88041	0,380167
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TNF & % other	114	-0,086554	-1,02061	0,309224

* - <0.05 relationships are valid

extracts was measured using a Qubit 3 fluorometer and with a set of Qubit dsDNA HS Assay Kits (Thermo Fisher Scientific) and adjusted to ~ 10 ng / µl. Determination of the composition of the IM at the level of major phylotypes was performed by identifying total bacterial DNA and DNA of *Bacteroidetes, Firmicutes* and *Actinobacteria* by quantitative polymerase chain reaction (PCR) in real time (qRT-PCR) using universal primers for the 16S rRNA gene and taxon-specific primers of production (Thermo Fisher Scientific) [19].

In addition to the standard examination, all patients were quantified by the composition of the colon microbiota by polymerase chain reaction (PCR) with hybridization-fluorescence detection of results in real time using a test system "Colonoflor-16", manufactured by "Alphalab".

STATISTICAL ANALYSIS

Statistical processing was performed using the package 'STATISTICA 13.1'. According to the Kolmogorov-Smirnov criterion, the distribution of all studied indicators was different from normal (Gaussian), so data processing was performed using non-parametric statistics. Further data are presented in the form of Me [LQ; UQ], where Me is the median (50 quartiles), and LQ and UQ are the lower and upper quartiles (25 and 75 percentiles, respectively). The dependence of the indicator on the group was investigated using the Kraskel-Wallace test and the Spearman correlation coefficient.

RESULTS

THE COMPOSITION OF INTESTINAL MICROBIOTA, THE RATIO OF THE MAIN FILES OF PATIENTS WITH NAFLD AND HEALTHY PEOPLE

To assess the state of the intestinal microbiota of patients with NAFLD, the composition of the IM was determined at the level of basic phylotypes by identifying total bacterial DNA and DNA of *Bacteroidetes*, *Firmicutes* and *Actinobacteria* and the data were compared to the obtained control indicators.

The obtained results are presented in fig 1.

In addition, significant changes were observed in the index of the *Firmicutes* / *Bacteroidetes*. This indicator was significantly higher than in the control group of patients with NAFLD and was 6.43 and 1.26 ($p < 0.05$), respectively.

THE COMPOSITION OF INTESTINAL MICROBIOTIA, THE RATIO OF MAIN FILES OF PATIENTS WITH NAFLD DEPENDING ON THE QUANTITY AND DISTRIBUTION OF ADIPOSE TISSUE

A detailed analysis of the obtained data showed that in patients with varying degrees of obesity there was a significant decrease in *Bacteroidetes* to 16.6 [8,3; 22.4]%, with moderate obesity and up to 10.6 [6.5; 19.1]% in morbid obesity with a simultaneous increase in the ratio of *Firmicutes* / *Bacteroidetes* to 3.1 [1.7; 6.2] and 5.4 [2.8; 7.5], respectively.

At the same time in patients with overweight these changes were a trend. The relative amount of *Actinobacteri*a did not differ in any of the examined groups. As the weight increases, there could be seen deeper changes in the ratio of the main bacterial fillets of IM.

In the future, we studied the dependence of IM changes on the amount and activity of VAT. The distribution of the main IM branches from the percentage of VAT are presented in fig 2.

In patients with NAFLD, as the percentage of VAT increased, there was a redistribution of IM in the direction of increasing *Firmicutes* compared with the control group and patients with NAFLD with a normal amount of VAT. At the same time, the number of *Bacteroidetes* decreased in the group of NAFLD with visceral obesity, while the content of *Actinobacteria* did not significantly change.

Taking into account the pathogenetic role of visceral fat in the formation of NAFLD, we studied the associations between indicators of VAT activity and the quantitative composition of IM. The data are shown in Fig 3.

The obtained data demonstrate a similar dependence of the distribution of the main IM cells on the activity of VAT. In patients with a high index of visceral obesity (IVO) there was a probable increase in the content of *Firmicutes bacteria*, in contrast, the number of *Bacteroidetes decreased* in these patients. In the group of patients with NAFLD with a low level of IVO, high variability in the composition of *Firmicutes* bacteria was found, although in general the group showed a tendency to increase the content of bacteria of this class.

These changes indicate the probable participation of bacteria of the *Firmicutes* genus in the formation of VAT, increasing its activity with the further development and progression of NAFLD.

We analyzed the dependence of the main IM fillets in patients with NAFLD on the degree of hepatic steatosis. The obtained data are presented in Fig 4.

The study revealed an imbalance of IM of patients with NAFLD with varying degrees of fatty infiltration of the liver compared with the control group. Namely, in patients with third grade of hepatic steatosis, maximal changes were observed, which was accompanied by inhibition of the growth of bacteria of the *Bacteroidetes* class, with a simultaneous increase in the content of *Firmicutes* ($p < 0.05$). Similar changes were observed in the group of patients with low and moderate steatosis, but the differences were tendentious.

THE RELATIONSHIPS BETWEEN INTESTINAL MICROBIOTA AND SYSTEMIC INFLAMMATION IN PATIENTS WITH NAFLD AGAINST COMORBID CONDITIONS

Markers of systemic inflammation were identified in all examined patients, namely tumor necrosis factor (TNF) -alpha, highly sensitive C-reactive protein (CRP), the data are shown in Fig 5.

When analyzing the concentrations of markers of systemic inflammation in the serum of patients with NAFLD compared with the control group significantly elevated levels of CRP and TNF-alpha ($p < 0.05$) were obtained indicating the pathogenetic role of inflammation in the development and progression of the disease. We analyzed the dependence of the main IM fillets from markers of systemic inflammation. The obtained data are shown in table I.

When comparing the levels of proinflammatory markers with the main IM phyla, a direct correlation dependence of weak degree of CRP and TNF alpha with *Firmicutes* content ($p < 0.05$) and an inverse correlation dependence of CRP with *Bacteroidetes* content ($p < 0.001$) were revealed.

In order to determine the influence of metabolic factors on the composition of IM and the activity of inflammation, the relationship of IM with markers of systemic inflammation in groups of patients with NAFLD with overweight and obesity was evaluated. The results are presented in table II.

We studied the dependence of the ratio of major intestinal fillets (*Firmicutes / Bacteroidetes*) from markers of systemic inflammation of patients with NAFLD with varying degrees of steatosis. It has been shown that there is a direct correlation between the presence of adipose tissue and the level of hepatic steatosis and pro-inflammatory markers (CRP and TNF-alpha), and one can indicate the development of IM in the process of accumulation of systemic chronic adipose of the liver tissue, which is more accordant with ailments with a high level of visceral obesity activity (fig 6).

It has been shown that in patients with NAFLD and a high degree of steatosis there were more significant changes in the main intestinal fillets in the direction of increasing the content of *Firmicutes*. High levels of TNF and CRP also affected the composition of IM, namely in these groups of patients an imbalance of the IM was detected, which was manifested by an increase in the *Firmicutes / Bacteroidetes* index. However, the identified changes were a trend.

DISCUSSION

Numerous studies have shown that altered IM can affect liver function in some way, causing inflammation, insulin resistance, and fat accumulation, which is also related to NAFLD [20].

Differences at the taxonomic level between IM of healthy people and patients with NAFLD can be multidirectional: conducive for increased intestinal microbiota resistance, or if continuous external influences are stressful and destructive, they can lead to unstructured microbiome, and dysbacteriosis in it's turn can contribute to disease progression [21].

The two largest phylotypes that make up the human intestinal microbiota are *Firmicutes* and *Bacteroidetes*, and to a less extent other phylotypes are represented: *actinobacteria, proteobacteria, fusobacteria* and *verrucomicrobial* medications [22]. The *Firmicutes / Bacteroidetes* ratio is associated with a number of pathological conditions. In particular, obesity was specifically associated with a greater number of *Firmicutes* and / or a decrease in *Bacteroidetes* (ie, an increase in the ratio); however, some studies did not show

any changes or even increases in bacteroid content [21, 23].

We consider the ratio *Firmicutes* / *Bacteroidetes* as an integral indicator, which best characterizes the violation of the relative composition of the microbiota at the level of basic phylotypes. However, the assessment of this ratio in some cases may require additional research, because the IM is not constant and shows significant heterogeneity within one phylotype [24, 25]. It should be noted that the *Firmicutes* / *Bacteroidetes* ratio increases from birth to adulthood and subsequently changes with aging. This ratio varies significantly between infants, adults and the elderly. This may be due to general changes in bacterial profiles at different stages of life [26].

Analysis of the literature shows a close relationship between the growth of the *Firmicutes* / *Bacteroidetes* index and the presence of metabolic disorders. Significantly higher values of this indicator are observed in obese people and obese mice (ob / ob) compared with the control group of lean people [27, 28]. The microbiota of obese people also had less bacterial diversity than in lean people [29]. In addition, when obese people lost weight using a low-fat or low-carbohydrate, low-calorie diet, the *Firmicutes* / *Bacteroidetes* ratio decreased due to a percentage reduction in body weight [23].

However, other studies on humans and rodents have not showed differences in this index in obese individuals compared to lean people and under the influence of weight loss, or even demonstrated its reduction in obese individuals [30]. The reason for these conflicting observations regarding the *Firmicutes* / *Bacteroidetes* index and obesity is currently unclear.

This may be an indication that the change in IM may occur unevenly for each taxon in the same phylotype. In addition, the results which were obtained are not consistent in different studies due to the small number of people and differences in age, gender, ethnicity, geographical location and medication use [31-43].

Undoubtedly, the IM plays a significant role in the pathogenesis and progression of NAFLD, which makes it extremely important to study the mechanisms of its influence on the homeostasis of the intestine and liver.

According to the modern literature, there are ambiguous research results on changes in the IM of patients with NAFLD in overweight and varying degrees of obesity. We analyzed the two largest phylotypes that make up the human IM - *Firmicutes* and *Bacteroidete*s, depending on BMI.

During the study of the relative quantitative composition of IM there were revealed significant differences in patients of examined group with NAFLD with overweight and varying degrees of obesity compared with the control group and the group of patients with NAFLD with normal weight. Thus, in all patients with NAFLD with normal weight, the distribution of the main microbial fillets did not differ from the control group. At the same time, in patients with NAFLD with overweight and obesity, there was a shift in the ratio of the main fillets towards an increase in *Firmicutes* and a decrease in *Bacteroidetes*, which led to an increase in the index of *Firmicutes* / *Bacteroidetes*.

The obtained data indicate the involvement of IM in the processes of systemic chronic inflammation, molecular mechanisms of ectopic fat deposition, in particular the formation of fatty degeneration of the liver, which is more evident in patients with a high degree of visceral obesity. IM imbalance leads to excessive synthesis of pro-inflammatory cytokines, promotes the activation of cellular mechanisms, which increases the flow of fatty acids into hepatocytes and increases the degree of hepatic steatosis.

CONCLUSIONS

1. In comparison with healthy individuals, patients with NAFLD showed a significant decrease in the relative content of *Bacteroidetes* with a simultaneous increase in the content of *Firmicutes* and an increase in the index of *Firmicutes* / *Bacteroidetes* ($p < 0.05$).
2. Overweight and obese NAFLD patients had a more significant IM imbalance in the form of an increase in the *Firmicutes* / *Bacteroidetes* index, due to inhibition of *Bacteroidetes* growth, compared with patients with normal BMI.
3. The revealed changes of the main IM fillets of the examined patients were observed not only with the increase of body weight, but also depended on the number and activity of VAT. The most significant changes were detected at high activity of VAT.
4. Deviations in the composition of IM, namely inhibition of the growth of bacteria of the class *Bacteroidetes* and the growth of the microflora *Firmicutes*, had an impact on the formation and severity of fatty infiltration of hepatocytes. Maximum changes in IM were observed in patients with a high degree of hepatic steatosis.
5. It was defined the weak direct correlation between the content of *Firmicutes* and proinflammatory markers (CRP and TNF alpha) and inverse correlation of CRP with the content of *Bacteroidetes*.
6. No associations were found between the composition of IM and inflammatory activity of patients with NAFLD with overweight and obesity, which indicates the absence of a significant effect of abdominal obesity on the state of IM.
7. The dependence of the ratio of the main intestinal phyla (*Firmicutes* / *Bacteroidetes*) on the markers of systemic inflammation and the activity of VAT was detected. Maximum index values (*Firmicutes* / *Bacteroidetes*) were observed in patients with NAFLD with a high degree of the activity of visceral obesity and elevated levels of CRP and TNF alpha.
8. Significant changes in the main intestinal phyla in the direction of increasing the content of *Firmicute*s were observed in patients with NAFLD with a high degree of IVO and elevated levels of proinflammatory cytokines.

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The Authors declare no conflict of interest.

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