EXPERIMENTAL WORKS

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DEPENDENCE OF INTESTINAL MICROBIOTA COMPOSITION ON DISTRIBUTION AND ACTIVITY OF ADIPOSE TISSUE IN NONALCOHOLIC FATTY LIVER DISEASE

Objective. *Nonalcoholic fatty liver disease (NAFLD) pathogenesis displays a close relation with intestinal dysbiosis. Thus, the aim of this study was to investigate the intestinal microbiota (IM) composition and to determine the correlation of changes in its main phylotypes with the amount and activity of adipose tissue in NAFLD patients.* ***Methods.*** *The prospective study enrolled 114 NAFLD patients with metabolic disorders and 30 healthy subjects as the control group. Along with routine examination, the authors assessed intestinal microbiota composition by identifying total bacterial DNA and DNA of Bacteroidetes, Firmicutes, and Actinobacteria by means of a quantitative real-time PCR.* ***Results.*** *NAFLD patients showed a significant decrease in the relative amount of Bacteroidetes with a simultaneous increase in the Firmicutes and an increase in Firmicutes/Bacteroidetes ratio compared with healthy subjects (p<0.05). NAFLD patients with concomitant overweight and obesity displayed a more significant imbalance of IM with an increase in the Firmicutes/Bacteroidetes ratio due to the inhibition of Bacteroidetes, compared with patients of normal body mass index. The revealed changes in the main phylotypes of IM in the examined patients were proven linked not only to an increase in body weight but also to the amount and activity of visceral adipose tissue. Furthermore, deviations in the gut microbiota composition had an impact on the formation and severity of steatosis.* ***Conclusions.*** *The study revealed an imbalance of IM in NAFLD patients. Further research in gut microbiota will help to elucidate their role in NAFLD pathogenesis and to lay a foundation for the development of individualized treatment.*

Keywords: Nonalcoholic fatty liver disease, Bacteroidetes, Firmicutes, Actinobacteria.

Today, nonalcoholic fatty liver disease (NAFLD) is regarded as a wide range of diseases caused by various dietary, metabolic, genetic, environmental, and microbiotic factors. A number of experimental and clinical studies have demonstrated evidence of a close relation between NAFLD, i.e. its progressive stage of nonalcoholic steatohepatitis (NASH), and intestinal dysbiosis [1, 2]. In this vein, the study of intestinal microbiota and its role in the pathogenesis of NAFLD acquires critical importance.

Noteworthily, the relationship between intestinal microbiota and host health has been proven in many chronic diseases, including obesity, diabetes, inflammatory bowel disease, colon cancer, etc.

It stands to reason that research into intestinal microbiota could lead to the development of a new arsenal to combat the devastating pandemic of obesity and its comorbidities that are increasingly threatening human society.

Trillions of microorganisms that colonize the human body, including bacteria, archaea, viruses, and eukaryotes, spread along the length of the gastrointestinal tract [3]. The equilibrium of key bacterial genera accounts for the healthy state of the intestinal microbiota [4]. The composition of the intestinal microbiota varies depending on the lumen and the intestinal mucosa. Common luminous microbial genera include *Bacteroides, Streptococcus, Bifidobacterium, Enterobacteriaceae, Enterococcus, Clostridium, Ruminococcus,* and *Lactobacillus.* Likewise, *Lactobacillus, Enterococcus,* and *Akkermansia* are more common in the mucus layer and the epithelial crypts of small intestine. Numerous factors can affect the composition and function of the intestinal microbiota. As shown, these include genetic triggers, diet, method of delivery at childbirth, geographical location, drug treatment, etc. [5, 6]. As a result, the intestinal microbiota is unique to any person and at the same time changes under various factors throughout the human life. In turn, the intestinal microbiota affects the host metabolic phenotype, participates in food and drug metabolism and regulates the immune system [7].

Latest research in intestinal microbiota substantiates new prospects for personalized medicine [8]. Advanced knowledge of the intestinal microbiota may prove even more importance in the development of personalized disease risk stratification models in clinical practice than metagenomics, metabolomics, metatranscriptomics, and metaproteomics [4].

This fully applies to liver disease. The intestine and liver are closely linked through anatomical and functional interactions via the portal venous system [9]. The portal vein supplies 70% of the total amount of blood in the liver, thus exposing the liver to intestine-related factors, such as nutrients and metabolites required for healthy homeostasis. Conversely, the intestinal microbiota can deliver detrimental, particularly to the liver, products such as endotoxins, peptidoglycans and even full-fledged bacteria leading to deep dysregulation of a number of hepatic metabolic pathways.

There is a growing body of evidence for the role of intestinal microbiota in the onset and development of liver disease, namely through the production of bioactive metabolites. In particular, the metabolomics research indicates that butyrate is a powerful metabolic and an inflammatory hepatic modulator. Some aromatic amino acids, such as phenylacetic acid, imidazole propionate, and 3- (4-hydroxyphenyl) lactate, have been identified as potential inducers of steatosis and hepatic inflammation, whereas indoline compounds (indole and indole-3-acetate) secures a healthy hepatic structure and functioning [10].

The strategy to demonstrate the causal role of the intestinal microbiota in the NAFLD pathogenesis of NAFLD lies in the investigating the association of the entire microbiome and analyzing potentially key intestinal microbial phylotypes linked to the etiology and/or develop-

ment of a particular chronic disease. Basically, researchers differentiate six key phylotypes in the intestinal microbiota. i.e. *Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Actinobacteria,* and *Fusobacteria* [11]. These bacteria can be involved in a variety of metabolism-affecting processes, including regulating polysaccharide levels, bile acid production, choline turnover, energy intake, stimulating endogenous ethanol synthesis, and protection against pathogens. Thus, the microbiota sustains intestinal homeostasis. Despite numerous beneficial effects of the intestinal microbiota on the host homeostasis, excessive proliferation of specific species can lead to overproduction of certain metabolites adversely affecting the gastrointestinal tract and even provoking systemic inflammation in the worst-case scenario [12].

The microbiota can improve or deteriorate the course of NAFLD through several mechanisms, including altered intestinal permeability, modulating energy intake, changing gene expression in de novo lipogenesis, choline and bile acid signaling pathways, producing ethanol in the gut and interacting with it. However, the associations between these factors and the development and/or progression of NAFLD still remain controversial.

Although recent studies in humans and animals have shown an association between intestinal dysbiosis and NAFLD, many questions remain open [13].

Currently, the efforts of many researchers are aimed at elucidating the molecular mechanisms that link changes in key bacteria in the gut with the cascade of host reactions, which ultimately leads to the endpoints of the disease.

Thus, intestinal microbiome communication plays a significant role in the development and progression of NAFLD and NASH. With a rapid increase in the incidence of NAFLD, the need for new preventive or therapeutic strategies based on the results of microbiota research becomes crucial.

All these taken into account, the **aim** of this study was to investigate the intestinal microbiota composition and to determine correlation of changes in its main phylotypes with the amount and activity of adipose tissue in NAFLD patients.

Materials and Methods. The study enrolled 114 NAFLD patients with metabolic disorders and 30 healthy subjects as the control group, all examined at the Department of Gastroenterology and Therapy and the Outpatient Department of the Governmental Institution "L.T. Malaya National Institute of Therapy of the National Academy of Medical Sciences of Ukraine". The mean age of the NAFLD patients was (52.56 ± 11.7) years.

Estimation of anthropometric parameters included measurement of height and body weight with a calculation of the body mass index (BMI). The distribution of adipose tissue was assessed by measuring the waist (WC) and hips circumferences (HC) with calculating the WC/HC ratio. To study the body composition of patients, determination of the total percentage of body fat, the percentage of visceral adipose tissue (VAT), we used an electronic device, a body composition scale-monitor OMRON BF-511. To determine the VAT dysfunction, we calculated the index of visceral obesity (IVO) by the Amato method [14].

Determination of the intestinal microbiota composition at the level of its main phylotypes was performed by identifying total bacterial DNA and DNA of *Bacteroidetes, Firmicutes,* and *Actinobacteria* by means of a quantitative polymerase chain reaction (PCR) in real time using universal primers for gene 16S rRNA and taxon-specific primers (Applied Biosystem) [15]. DNA was extracted from feaces with the use of the reagent kit "Ribo-prep nucleic acid extraction kit" (AmpliSens). The DNA concentration in the extracts was measured using a Qubit 3 fluorometer and a reagent kit "Qubit dsDNA HS Assay Kits" (Thermo Fisher Scientific). Amplification was performed with the detection system CFX96Touch (Bio-Rad, USA).
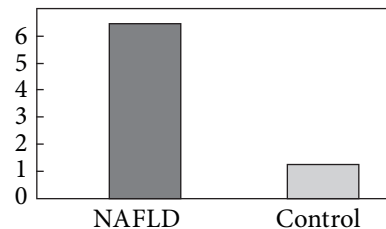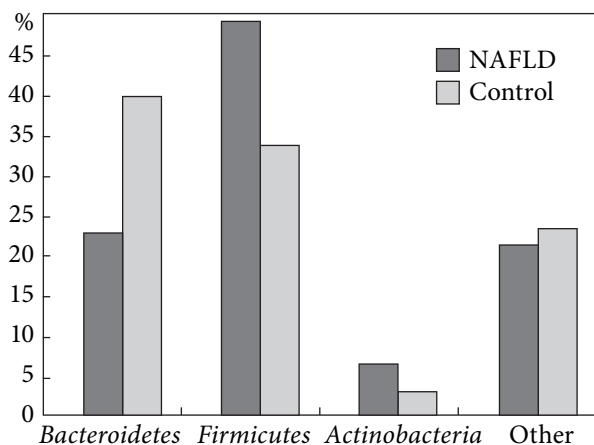
Fig. 2. *Firmicutes/Bacteroidetes* ratio in NAFLD patients and healthy subjects

◄ **Fig. 1.** Relative composition of IM at the level of its main phylotypes in NAFLD patients against the control group
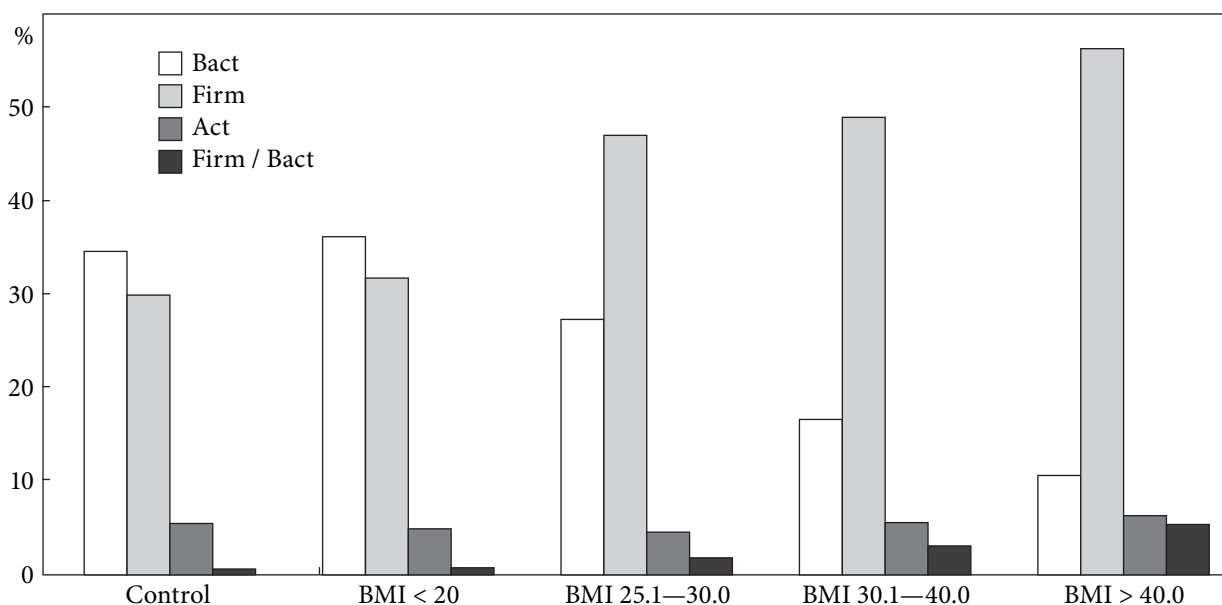

Fig. 3. The relative composition of IM at the level of its main phylotypes in NAFLD patients with different BMI; * p < 0.05 — statistically significant changes when compared with the control group and NAFLD patients with normal weight

The analysis of the results was performed with the computer program SPSS 21 for Windows XP via primary descriptive statistics, Student's t-test for dependent and independent samples, and correlation analysis.

Results. To assess the intestinal microbiota (IM) composition in NAFLD patient at the level of its main phylotypes, we identified total bacterial DNA and DNA of *Bacteroidetes, Firmicutes,* and *Actinobacteria* and compared the obtained data with controls. The results obtained are presented in Fig. 1.

NAFLD patients showed a statistically significant increase in *Firmicutes* bacteria with a simultaneous decrease in *Bacteroidetes* and a slight increase in *Actinobacteria*. Furthermore, significant changes were observed in the *Firmicutes: Bacteroidetes* ratio (Fig. 2). This was significantly higher in NAFLD patients compared with the control group (6.43 vs 1.26, respectively, p <0.05).

We regard the *Firmicutes/Bacteroidetes* ratio as an integral index, which best characterizes the changes in a relative composition of the IM at the level of its main phylotypes. However, the assessment of this ratio in some cases may require further research because the IM is not constant and shows significant heterogeneity within a single phylotype.

Modern literature is indicative of ambiguous results as to changes in IM in NAFLD patients with concomitant overweight and/or obesity.

We analyzed the two largest phylotypes of the human IM, i.e. *Firmicutes* and *Bacteroidetes*, depending on BMI, as shown in Fig. 3.

The study of the relative quantitative composition of IM in the examined groups revealed significant differences in NAFLD patients with concomitant overweight and/or obesity compared with the control group and the group of NAFLD patients of normal weight. All NAFLD patients of normal weight did not show any differences in the distribution of the main microbial phylotypes when compared with the control group. At the same time, NAFLD patients with concomitant overweight and/or obesity demonstrated a shift in the ratio of the main phylotypes towards an increase in *Firmicutes* and a decrease in *Bacteroidetes* leading to an increase in the *Firmicutes/Bacteroidetes* ratio. A detailed analysis of the obtained data determined that NAFLD patients with moderate obesity showed a significant decrease in *Bacteroidetes* to 16.6 (8.3; 22.4) %, 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. However, these changes in overweight patients were only tendentious. Likewise, we did not find any significant changes in *Actinobacteria* in all examined groups. As the weight increases, there are deeper changes in the ratio of the main bacterial phylotypes of IM.

Next, we studied the dependence of IM composition on the amount and activity of VAT as presented in Fig. 4.
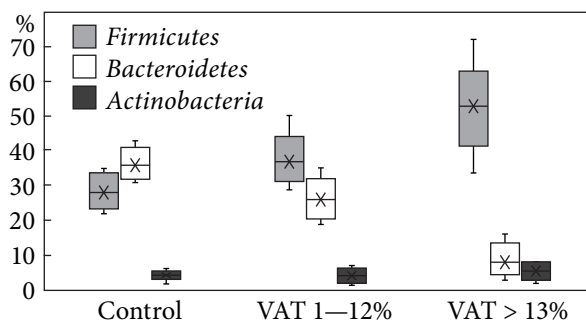
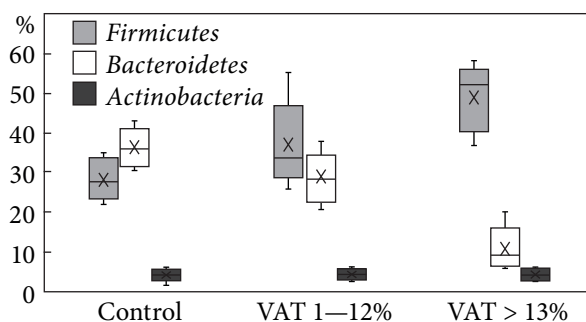**Fig. 4.** Distribution of the main phylotypes of IM in NAFLD patients depending on the VAT (%)

Fig. 5. Changes in the IM composition in NAFLD patients depending on the VAT activity

As the VAT increased, there was a redistribution of IM composition with an increase in *Firmicutes* in comparison with the control group and NAFLD patients of normal VAT. At the same time, the number of *Bacteroidetes* decreased in NAFLD patients with concomitant visceral obesity, while the content of *Actinobacteria* did not change significantly.

Given the pathogenic role of visceral fat in NAFLD formation, we studied the relation between VAT activity and the quantitative composition of IM, as shown in Fig. 5.

The obtained data show a similar dependence of the distribution of the main IM phylotypes on the VAT activity. Patients with a high IVO showed a significant increase in *Firmicutes* and simultanious decrease *in* the number of *Bacteroidetes*. NAFLD patients with a low IVO displayed a high variability in the composition of
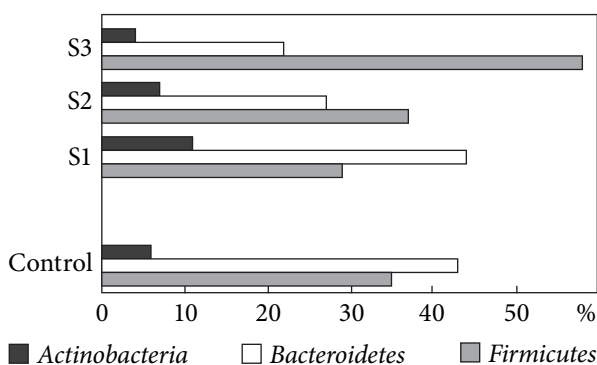
Fig. 6. Main phylotypes of IM in NAFLD patients depending on the hepatic steatosis degree: S1 — grade 1 steatosis; S2 — grade 2 steatosis; S3 — grade 3 steatosis

Firmicute with a tendency to general increase in this class of bacteria.

These changes are indicative the possible role of *Firmicutes* in the VAT formation and increase in VAT activity with a subsequent NAFLD evolvement and progression.

Recently, IM has been shown to play a key role in the NAFLD evolvement and progression. Deviations in the IM composition lead to oxidative stress, metabolic disorders, chronic low-grade inflammation, particularly in liver tissue. We analyzed the correlation of the main phylotypes of IM in NAFLD patients with hepatic steatosis, as presented in Fig. 6.

We revealed an imbalance of IM composition in NAFLD patients with different steatosis degrees compared with the control group. Patients with advanced steatosis showed the maximum changes, which were accompanied by inhibition of the *Bacteroidetes* bacterial phylum with a simultaneous increase in the content of *Firmicutes*. Similar changes were observed in the group of patients with a low or moderate steatosis, but the differences were tendentious.

Discussion. Note-worthily, the *Firmicutes / Bacteroidetes* ratio increases from birth to adulthood and subsequently changes with ageing. Due to general changes in bacterial profiles at different stages of life [16], this ratio varies significantly with age.

Although no specific therapeutic interventions for a markedly elevated or depressive *Firmicutes/Bacteroidetes* ratio are yet known, general medication strategies can affect this ratio and ultimately modulate intestinal health.

Our data support the results of the study by Mouzaki et al. who recruited a group of 50 adults: 11 with biopsy-proven 'simple' steatosis, 22 with biopsy-proven NASH, and 17 healthy controls with no steatosis by biopsy [12] and showed significantly decreased levels of *Bacteroidetes* in NASH compared to simple steatosis and controls, even after adjusting for body mass index. Furthermore, our results correlate with those obtained by Raman et al. in a cross-sectional study of 60 adults: 30 obese individuals with clinically defined NAFLD (no biopsy available) and 30 non-obese controls [17]. The authors found an increase in the phylum Firmicutes in NAFLD, compared with non-obese controls as well as elevation in levels of hepatotoxic volatile organic compounds, which have been shown to promote the development of NAFLD.

Latest research substantiates a close relation between an increase in the *Firmicutes/Bacteroidetes* index and metabolic disorders. Significantly higher values of the *Firmicutes/Bacteroidetes* ratio were observed in patients living with obesity and obese mice (ob/ob) compared with the control group of normal weight [13]. Patients living with obesity displayed less bacterial diversity of intestinal microbiota, compared with subjects of normal weight [18]. Furthermore, weight-loss due to low-fat, low-carbohydrate or low-calorie diets was proven associated with a decrease in the *Firmicutes/Bacteroidetes* ratio in patients living with obesity [19].

However, other studies in humans and rodents have not found significant differences in this ratio in people living with obesity, compared with subjects of normal weight, under the influence of weight loss or even demonstrated its reduction [20]. The cause of these confronting observations regarding the connection between

the *Firmicutes/Bacteroidetes* ratio and obesity is currently unclear.

There is evidence that bacteria associated with metabolic disorders and obesity induce the expression of genes that regulate lipid and carbohydrate metabolism; this can lead to an increased absorption of energy-valuable substances from the diet [21].

A number of researchers tend to link in one way or another metabolic-associated diseases and changes in intestinal microbiota. A study of the results of sequencing bacterial 16S rRNA sequences in 154 homo- and heterozygous twins and their mothers, including patients living with obesity and subjects of normal weight, revealed an association of obesity with a decrease in the phylogenetic diversity in intestinal microbiota, a reduction in *Bacteroidetes* and an increase in *Actinobacteria* [22]. Further analysis of 383 different microbiota genes in the obese and lean gut microbiome showed that 75 % of the genes associated with obesity belonged to *Actinobacteria* with the other 25 % to *Firmicutes*. At the same time, 42 % of genes associated with normal weight were found in *Bacteroidetes* [23].

In addition to the genera *Firmicutes/Bacteroidetes* ratio, there is evidence of the influence of *Bifidobacterium* spp. on the development of obesity; namely, their volume reduced in children living with obesity compared with their peers of normal weight. Note-worthily, bacteria of different species within the same phylotype can have different effects on the physiological processes, so it is advisable to continue research into specific correlations within separate bacterial species.

NAFLD patients showed a significant difference in the *Bacteroides* and *Prevotella* in the faeces compared with healthy volunteers [24]. In another study, the proportion of *Bacteroides* and *Prevotella* was inversely related to NAFLD [11]. The study also identified an independent association between *Bacteroides* and NAFLD, as well as between *Ruminococcus* and advanced fibrosis. The severity of NAFLD was closely related

to intestinal dysbiosis and changes in the metabolic function of the microbiota. Streptozotocin and high fat diets (NASH) were associated with an increase in *Bacteroides acidifaciens, B. uniformis, B. vulgatus, Clostridium cocleatum,* and *C. xylanolyticum*. NAFLD patients showed an increased intestinal permeability and an increase in the number of gamma-proteobacteria as well as *Bacteroidetes,* compared with healthy individuals.

Undoubtedly, the intestinal microbiota plays a significant role in the NAFLD pathogenesis, which makes it crucial to investigate the mechanisms of its influence on the homeostasis of the intestine and liver. Further research in intestine microorganisms will help to elucidate their role in human metabolism in order to form a more accurate picture of the NAFLD pathogenesis and to lay a foundation for the development of individualized treatment.

Conclusions

1. NAFLD patients showed a significant decrease in the relative numder of *Bacteroidetes* with a simultaneous increase in the *Firmicutes* and an increase in *Firmicutes / Bacteroidetes* ratio, compared with healthy subjects (p<0.05).

2. NAFLD patients with concomitant overweight and obesity displayed a more significant imbalance of IM with an increase in the *Firmicutes/Bacteroidetes* ratio due to the inhibition of *Bacteroidetes,* compared with patients of normal BMI.

3. The revealed changes in the main phylotypes of IM in the examined patients were proven linked not only to an increase in body weight, but also to the amount and activity of VAT. The most significant changes were observed at VAT high activity levels.

4. Deviations in the IM composition, namely the reduction in the *Bacteroidetes* phylum and an increase in *Firmicutes*, had an impact on the formation and severity of steatosis. Maximum changes in IM were observed in patients with an advanced hepatic steatosis.

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ЗАЛЕЖНІСТЬ СКЛАДУ КИШКОВОЇ МІКРОБІОТИ ВІД РОЗПОДІЛУ ТА АКТИВНОСТІ ЖИРОВОЇ ТКАНИНИ ПРИ НЕАЛКОГОЛЬНІЙ ЖИРОВІЙ ХВОРОБІ ПЕЧІНКИ

Мета дослідження. Неалкогольна жирова хвороба печінки (НАЖХП) — це спектр захворювання, який обумовлений різними механізмами, що включають дієтичні, метаболічні, генетичні, екологічні та мікробіотичні фактори. Ряд експериментальних та клінічних досліджень продемонстрували докази тісного взаємозв'язку НАЖХП і неалкогольного стеатогепатиту (НАСГ) з дисбактеріозом кишечника. На даний час дослідження мікробіоти та її ролі в патогенезі НАЖХП набуло надзвичайної актуальності. **Методи.** До проспективного дослідження було зараховано 114 пацієнтів з НАЖХП з порушенням обміну речовин та 30 здорових суб'єктів в якості контрольної групи. Поряд із плановим обстеженням автори оцінювали склад кишкової мікробіоти (КМ) шляхом ідентифікації загальної бактеріальної ДНК та ДНК бактероїдів, фірмікутів та актинобактерій за допомогою кількісної ПЛР у реальному часі. **Результати.** У хворих на НАЖХП спостерігалось вірогідне підвищення вмісту бактерій Firmicutes з одночасним зниженням кількості Bacteroidetes та незначне підвищення вмісту Actinobacteria. Крім того, суттєві зміни відзначалися і у значенні індексу *Firmicutes:Bacteroidetes*. У хворих на НАЖХП цей показник був значно вищим, ніж в контрольній групі (p < 0,05) та становив відповідно 6,43 та 1,26 . Проаналізовано два найбільших філотипи — *Firmicutes* та *Bacteroidetes*, в залежності від ІМТ. У всіх хворих на НАЖХП з нормальною вагою розподіл основних мікробних філів не відрізнявся від групи контролю, тоді як у хворих з надлишковою вагою та ожирінням спостерігався зсув співвідношення основних філів у бік збільшення *Firmicutes* та зменшення *Bacteroidetes,* що призводило до зростання індексу *Firmicutes/Bacteroidetes*. При детальному аналізі отриманих даних визначено, що у хворих з різними ступенями ожиріння спостерігалось достовірне зниження *Bacteroidetes* до 16,6 (8,3; 22,4) % при помірному ожирінні та до 10,6 (6,5; 19,1) % — при морбідному ожирінні з одночасним підвищенням співвідношення *Firmicutes/Bacteroidetes* до 3,1 (1,7; 6,2) та 5,4 (2,8; 7,5) співвідносно. У той же час у пацієнтів з надлишковою вагою ці зміни носили характер тенденції. Відносна кількість *Actinobacteria* майже не відрізнялась у жодній з обстежених груп. Зі зростанням ваги спостерігаються глибші зміни у співвідношенні основних бактеріальних філів КМ. У хворих з високим індексом вісцерального ожиріння спостерігалось вірогідне підвищення вмісту бактерій *Firmicutes*. і зниження кількості *Bacteroidetes*. У групі хворих з низьким індексом вісцерального ожиріння була виявлена висока варіабельність складу бактерій *Firmicutes*, хоча в цілому по групі визначалася тенденція до підвищення вмісту бактерій цього класу. Виявлений дисбаланс КМ у хворих на НАЖХП з різним ступенем жирової інфільтрації печінки у порівнянні з групою контролю, а саме у хворих з третім ступенем стеатозу печінки, спостерігались максимальні зміни, що супроводжувалось пригніченням росту бактерій класу *Bacteroidetes* з одночасним зростанням вмісту *Firmicutes*. У групі хворих з низьким та помірним ступенем стеатозу спостерігались подібні зміни, але відмінності носили характер тенденції. **Висновки.** У результаті дослідження виявлено дисбаланс КМ у хворих на НАЖХП. Подальші дослідження мікробіоти кишечника допоможуть з'ясувати їхню роль у патогенезі НАЖБП та закласти основу для розвитку індивідуального лікування.

Ключові слова: неалкогольна жирова хвороба печінки, Bacteroidetes, Firmicutes, Actinobacteria.