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# Gut microbiota changes in nonalcoholic fatty liver disease and concomitant coronary artery disease

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SOUHRN

**Kontext a cíl výzkumu:** Nealkoholické ztukovatění jater (nonalcoholic fatty liver disease, NAFLD) má závažné ekonomické dopady na zdravotnictví celosvětově a na Ukrajině obzvláště. Hlavní příčinou mortality pacientů s NAFLD jsou kardiovaskulární onemocnění (KVO). Za potenciální mechanismus rozvoje ischemické choroby srdeční (ICHS) u pacientů s NAFLD lze považovat změny ve složení střevní mikrobioty. ***Cílem našeho výzkumu*** bylo zjistit změny koncentrací hlavních fylotypů střevní mikrobioty, kmenů *Bacteroidetes*, *Firmicutes* a *Actinobacteria*, a kvantifikovat koncentrace kmenů *Firmicutes*/*Bacteroidetes* u pacientů s NAFLD a současně s ICHS.
**Materiál a metody:** Do studie bylo zařazeno 109 jedinců s NAFLD (25 současně s arteriální hypertenzí [AH] a 24 současně s ICHS). Složení střevní mikrobioty bylo hodnoceno metodou qPCR.
**Výsledky a závěry:** U obou podskupin, s ICHS a s AH jako komorbiditami, byl pozorován výrazný trend ke zvyšování koncentrací *Bacteroidetes* (o 37,11 %, resp. 21,30 %) a snižování koncentrací kmene *Firmicutes* (o 7,38 %, resp. 7,77 %), přičemž nalezené změny nedosahovaly statistické významnosti. Ve srovnání s pacienty pouze s NAFLD bylo u nemocných s NAFLD plus ICHS zaznamenáno statisticky významné snížení koncentrací kmene *Actinobacteria* o 41,37 % ($p < 0,05$). U pacientů s NAFLD plus AH byly koncentrace kmene *Actinobacteria* nižší o 12,35 % (statisticky nevýznamný rozdíl). Byly nalezeny změny ve složení střevní mikrobioty, konkrétně nižší koncentrace kmene *Actinobacteria* u pacientů s ICHS; toto zjištění si vyžádá další výzkum.



*Keywords:*
Cardiovascular diseases
Coronary artery disease
Gut microbiota
Nonalcoholic fatty liver disease

ABSTRACT

**Background and the research aim:** Nonalcoholic fatty liver disease (NAFLD) bears serious economic consequences for the health care system worldwide and Ukraine, in particular. Cardiovascular diseases (CVD) are the main cause of mortality in NAFLD patients. Changes in the gut microbiota composition can be regarded as a potential mechanism of CVD in NAFLD patients. The ***research aim*** was the investigation changes in major gut microbiota phylotypes, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* with quantification of *Firmicutes*/*Bacteroidetes* in NAFLD patients with concomitant CVD.
**Materials and methods:** There were 109 NAFLD subjects (25 with concomitant arterial hypertension [AH] and 24 with coronary artery disease [CAD]) enrolled. The gut microbiota composition was assessed by qPCR.
**Results and conclusions:** There was a marked tendency towards an increase in the concentration of *Bacteroidetes* (by 37.11% and 21.30%, respectively) with a decrease in *Firmicutes* (by 7.38% and 7.77%, respectively) found in both groups with comorbid CAD and AH with the identified changes not reaching a statistical significance. A statistically significant decrease in the concentration of *Actinobacteria* was revealed in patients with NAFLD with concomitant CAD at 41.37% ($p < 0.05$) as compared with those with an isolated NAFLD. In patients with concomitant AH, the content of *Actinobacteria* dropped by 12.35%, which was statistically insignificant. There were changes found in the intestinal microbiota composition, namely decrease in *Actinobacteria* in patients with CAD, which requires further research.

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## Introduction

An excessive fat accumulation in hepatocytes with subsequent progressive inflammatory and fibrotic changes is a pathophysiological basis of most common liver pathologies such as nonalcoholic fatty liver disease (NAFLD).[1] In most cases, NAFLD shows a benign course, remaining at the stage of nonalcoholic steatosis (NAS). Still, sometimes NAS progresses to a more severe stage, including nonalcoholic steatohepatitis, cirrhosis, and hepatocellular carcinoma.[2] The significance of NAFLD in public health is substantiated by its multifaceted impact on morbidity and mortality, as well as economic[3] and social consequences associated with a shorter life expectancy and a burden of metabolic complications, including cardiovascular diseases (CVD).

CVD belong to the leading causes of mortality among patients with NAFLD.[1,4–6] A meta-analysis of 16 observational studies, which included a total of 34,043 NAFLD patients and 2,600 cardiovascular severe events over an average period of 6.9 years, showed an increase in the risk of fatal and/or cardiovascular non-fatal events (OR 1.64, 95% CI 1.26 to 2.13) which in turn increased as NAFLD progressed.[7] An increase in a cardiovascular risk in NAFLD patients is observable even in patients with a relatively low body mass index (BMI)[5,8] and persists with an allowance for common risk factors like fatty liver as well as severe fibrosis as independent risk factors for CVD.[9,10]

Both NAFLD and CVD are clinical manifestations of metabolic syndrome. Moreover, there is a specific NAFLD contribution to increasing cardiovascular (CV) risks. This can include common pathogenic mechanisms and risk factors such as being overweight with adipose tissue dysfunction, insulin resistance as well as impaired insulin signaling, atherogenic dyslipidemia, systemic inflammation, and altered gut microbiota composition.[11] The underlying mechanisms of connecting NAFLD to CVD are very complex and simultaneously involve a number of different pathways that are still not completely understood.[5]

Whereas the data on the role of intestinal microbiota in the development of atherosclerosis, hypertension, and heart failure[12–14] are abandoned, the research in NAFLD patients with concomitant CVD is still scarce, and the relation between the microbial composition and the development of cardiovascular pathology in patients within this category remains unclear.[15]

**The research aim** was the investigation of the changes in major gut microbiota phylotypes (*Bacteroidetes, Firmicutes,* and *Actinobacteria*) with quantification of *Firmicutes/Bacteroidetes* in NAFLD patients with concomitant CVD.

## Materials and methods

### *Subjects and methods*

The study was carried out at the Department for the Study of GI Diseases and their Comorbidity with Non-Communicable Diseases of the Governmental Institution "L.T. Malaya National Institute of Therapy of the National Academy of Medical Sciences of Ukraine" as part of the research grant from the UEG Working Group for Stool Banking and Fecal Microbiota Transplantation. The study enrolled 60 NAFLD outpatients and 49 NAFLD patients with concomitant CVD, i.e. 25 subjects with CAD, who represented the main group. The control group consisted of 20 healthy volunteers.

The study was approved by the Local Ethics Committee of the Institution (Protocol 04 from April 2019 and 6 from 17 July 2020) as carried out in compliance with the Law of Ukraine "On Medical Products" (1996); Principles of ICH GCP (2008); Ordinance of the Ministry of Health of Ukraine № 690 of September 23, 2009, "On Establishment of the Rules of Clinical Trials and Expert Examination of Clinical Trial Materials and the Standard Regulations on the Ethics Committee" with amendments. All patients signed informed consent for inclusion in the study.

The patients' examination was conducted in compliance with the recommendations of the European Association for the Study of the Liver (EASL);[16] the national adapted clinical guidelines "Non-alcoholic fatty liver disease" and "Non-alcoholic steatohepatitis". All patients underwent an interview to determine secondary fatty liver etiologic factors.

Patients with viral hepatitis B and C, hemochromatosis, autoimmune hepatitis, coeliac disease, and Wilson's disease were excluded from the study as well as patients with severe steatohepatitis and cirrhosis. Exclusion criteria were other conditions possibly affecting the composition of the gut microbiota like bacterial overgrowth syndrome, antibiotic or probiotic treatment.

The steatosis degree was evaluated according to the NAS index, and fibrosis degree according to the METAVIR scale with the hepatobiliary system ultrasound visualization (Soneus P7 ultrasound scanning system) by the wave attenuation coefficient and shear wave elastometry, respectively. The value of the wave attenuation coefficient from 1 to 2.2 dB/cm indicated the absence of steatosis, the value from 2.2 to 2.3 dB/cm – the first degree steatosis, from 2.3 to 2.9 dB/cm – the second degree steatosis and from 2.9 to 3.5 dB/cm – the third degree steatosis. In turn, the value of the shear wave elastometry coefficient from 0 to 5.8 kPa indicated the absence of fibrosis, from 5.8 to 7.0 kPa – fibrosis FI, from 7.0 to 9.5 kPa – F II, from 9.5 to 12.5 kPa – F III, and more than 12.5 kPa was indicative of F IV, or cirrhosis.

The CVD verification, in particular CAD and hypertension, was performed prior to the enrollment in the study, in compliance with ESC and ACC/AHA guidelines for the diagnosis and management of patients with stable coronary artery disease and arterial hypertension.[17,18] Twenty-five (22.94%) patients in the main group were diagnosed as having concomitant arterial hypertension (AH), and 24 (22.02%) patients were diagnosed as those with CAD.

The serum concentration of endotoxin (ET) was determined using the LAL Chromogenic Endpoint Assay kit (Hycult Biotech, Netherlands) with concentration range 0.01–10 endotoxin units/milliliter (U. ET/mL), sensitivity 0.01 U. ET/mL.

#### *Detection of the gut microbiota phylotypes*

The assessment of the major gut microbiota phylotypes was performed by identifying the total bacterial DNA and DNA of *Bacteroidetes, Firmicutes,* and *Actinobacteria* via

the real-time quantitative polymerase chain reaction.[19,20] Firstly, freshly collected faeces samples were aliquoted in sterile containers with the following quick freezing and storage until extraction at 20 °C. Next, DNA was extracted from 400 mg of faeces with the use of a Ribo-prep nucleic acid extraction kit (AmpliSens, Russian Federation). The DNA concentration in the extracts was measured with the use of a Qubit 3 fluorometer with a Qubit dsDNA HS Assay Kits (Thermo Scientific, USA) and rectified it to ~10 ng/μl. The PCR amplification and HRM analysis were performed using the real-time PCR product detection system, CFX96 Touch (Bio-Rad, USA). Amplification program included:

– initial stage of denaturation for 5 min at 95 °C – 40 cycles: 15 s at 95 °C, 15 s at 61.5 °C, 30 s at 72 °C with the reading of the fluorescence signal;
– the final stage of elongation – 5 min at 72 °C.

Quantitative composition of the colon microbiota was determined by the kit "Colonoflor-16" (Alfalab, Russian Federation) also via the real-time quantitative polymerase chain reaction with hybridization-fluorescence detection of results (CFX96Touch, Bio-Rad, USA). The number of microorganisms and their ratio were calculated using the kolonoflor_17_10.exe software.

Anthropometric measurements included determination of the anthropometric indices, i.e. height in metres; body weight in kilograms with the following quantification of the BMI and body composition with the use of bioimpedancemetry (OMRON BF 511, Japan, registration number № 20180102074). The distribution of adipose tissue was assessed by measuring the waist (WC) and hips (HC) circumferences and a quotient WC/HC. All patients were evaluated for their liver function, carbohydrate metabolism and lipid metabolism. Liver function was assessed by measuring the alanine and asparagine transaminases, alkaline phosphatase, and gamma-glutamyl transferase (automatic biochemical analyzer "HumaStar 200", registration number № 21150707005, Germany). Carbohydrate metabolism was evaluated through the serum glucose concentration (automatic biochemical analyzer "HumaStar 200", registration number №21150707005, Germany), glycosylated hemoglobin (automatic biochemical analyzer photometer "Humalyzer 2000", registration number № 18300, Germany), insulin (DRG Instruments GmbH, Germany) followed by a quantitative assessment of insulin resistance according to the HOMA-IR. Proinflammatory state was assessed via measurement serum C-reactive protein («CRP-BEST», LLC Best Diagnostic, Ukraine) and tumor necrosis α concentrations («EIA-TNF-alpha», Cytokine, Russia).

The bacterial overgrowth syndrome was excluded the hydrogen breath test (gas analyzer Gastro + Gastrolyzer, № 12-8989, UK).

The statistical analysis was performed with the use the software package 'STATISTICA 13.1' (Statsoft, USA). According to the Kolmogorov–Smirnov criterion, the distribution of all studied parameters was established as different from normal (Gaussian), and explained using non-parametric statistical methods for the data processment, hereinafter referred to as Me (LQ; UQ), where Me is the median, and LQ and UQ are the lower and upper quartiles, respectively. The dependence of the variables on the groups was investigated using the Kraskel–Wallace test.

## Results

### *Clinical characteristics*

Clinical characteristics of patients are shown in Table 1.

**Table 1 – Clinical characteristics of the examined patients**

| Parameter | Group 1<br>NAFLD patients | Group 2<br>NAFLD patients with concomitant CAD | Group 3<br>NAFLD patients with concomitant AH |
|---|---|---|---|
| | N = 60 | N = 24 | N = 25 |
| Age, years | 44.00<br>[37.00; 56.00] | 57.50<br>[48.5; 62.50]<br>$p_{1-2}$ <0.05 | 60.00<br>[49.00; 63.00]<br>$p_{1-3}$ <0.05 |
| BMI, kg/m$^2$ | 30.63<br>[29.20; 36.40] | 35.30<br>[31.60; 41.45] | 37.40<br>[31.80; 41.20]<br>$p_{1-3}$<0.05 |
| Waist circumference, cm | 110.00<br>[102.00; 122.00] | 116.00<br>[107.50; 123.50] | 116.00<br>[110.00; 128.00] |
| Total bilirubin, μmol/L | 9.22<br>[8.11; 13.61] | 9.10<br>[8.40; 14.50] | 9.20<br>[8.1; 11.90] |
| Direct bilirubin, μmol/L | 2.94<br>[2.31; 4.10] | 2.90<br>[2.10; 4.75] | 2.70<br>[2.10; 4.70] |
| ASAT, U/L | 25.00<br>[20.00; 37.00] | 26.5<br>[21.00; 33.00] | 27.00<br>[21.00; 33.00] |
| AlAT, U/L | 28.00<br>[20.50; 39.00] | 25.00<br>[21.00; 32.50] | 28.00<br>[20.00; 41.00] |
| GGT, U/L | 22.00<br>[12.00; 41.00] | 21.6<br>[16.5; 32.5] | 21.90<br>[14.60; 41.30] |

*Pokračování na další straně*

**Table 1 – Clinical characteristics of the examined patients *(Dokončení)***

| Parameter | Group 1<br>NAFLD patients | Group 2<br>NAFLD patients with concomitant CAD | Group 3<br>NAFLD patients with concomitant AH |
|---|---|---|---|
| | N = 60 | N = 24 | N = 25 |
| Alcaline phosphatase, U/L | 1461.00<br>[1278.00; 1720.00] | 1490.00<br>[1236.50; 1793.50] | 1457.00<br>[1254.00; 1794.00] |
| Total cholesterol, mmol/L | 5.25<br>[4.85; 5.92] | 5.42<br>[4.92; 6.16] | 5.81<br>[5.28; 6.45] |
| Triglycerides, mmol/L | 1.65<br>[1.11; 2.24] | 1.75<br>[1.30; 2.46] | 1.87<br>[1.08; 2.42] |
| VLDL, mmol/L | 0.79<br>[0.54; 1.09] | 0.82<br>[0.53; 1.06] | 0.84<br>[0.49; 1.13] |
| HDL, mmol/L | 1.28<br>[1.02; 1.48] | 1.18<br>[0.93; 1.32] | 1.17<br>[0.96; 1.34] |
| LDL, mmol/l | 3.02<br>[2.26; 3.81] | 3.16<br>[2.44; 3.75] | 3.54<br>[2.54; 4.04] |
| Glucose, mmol/L | 5.61<br>[5.09; 5.72] | 5.71<br>[5.28; 6.30] | 5.68<br>[5.22; 6.05] |
| $HbA_{1c}$, % | 6.09<br>[5.73; 6;81] | 6.07<br>[5.79; 7.39] | 6.07<br>[5.68; 7.24] |
| Insulin, μU/mL | 19.01<br>[16.41; 24.79] | 19.05<br>[16.68; 27.53] | 19.04<br>[16.94; 24.77] |
| HOMA-IR | 4.84<br>[3.68; 6.18] | 3.74<br>[3.91; 8.73] | 4.45<br>[4.04; 7.19] |
| CRP, mg/L | 6.91<br>[5.31; 8.27] | 8.63<br>[6.24; 11.08] | 9.06<br>[5.96; 10.87] |
| TNF-α, pg/mL | 6.49<br>[5.59; 7.51] | 7.71<br>[6.39; 9.03]<br>$p_{1-2}$ <0.05 | 7.28<br>[6.40; 8.41]<br>$p_{1-3}$ <0.05 |
| *Bacteroidetes*, % | 16.05<br>[8.47; 27.48] | 22.02<br>[7.20; 38.62] | 19.48<br>[6.94; 27.81] |
| *Firmicutes*, % | 61.84<br>[43.13; 66.12] | 49.61<br>[37.12; 60.98] | 49.39<br>[37.25; 61.82] |
| *Actinobacteria*, % | 5.12<br>[2.77; 9.81] | 2.99<br>[1.63; 5.95]<br>$p_{1-2}$ <0.05 | 4.47<br>[2.31; 7.62] |
| *Other*, % | 16.99<br>[10.26; 26.16] | 20.83<br>[7.16; 31.63] | 20.85<br>[7.86; 35.38] |
| *F/B* | 3.85<br>[1.43; 7.92] | 2.84<br>[1.15; 6.5] | 2.96<br>[1.71; 7.63] |

$p_{1-3}$ <0.05 – statistically significant difference between NAFLD patients and with concomitant AH.
$p_{1-2}$ <0.05 – statistically significant difference between NAFLD patients and with concomitant CAD.

The patient clinical examination showed an increase in BMI in all groups, with a statistical significance determined only in the AH group, in which BMI exceeded this parameter in CAD and isolated NAFLD patients by 4.82% and 22.10% ($p$ <0.05), respectively.

All patients displayed no statistically significant changes in liver function, lipid, and carbohydrate metabolism, which could be explained through the adequate therapy administered upon the case diagnosis. In particular, the cholesterol target values during the statin treatment were achieved in 8 patients (30.00%).

An increase in the concentration of pro-inflammatory factors was observed in both, AH and CAD, groups. Compared with isolated NAFLD patients, the CRP concentration proved an increase in CAD patients by 24.89%, and by 31.11% in AH patients, without reaching any statistical significance ($p$ = 0.05 and $p$ = 0.06, respectively). However, similar changes in the TNF-α concentration with an elevation by 18.79% in CAD patients and by 12.17% in the AH group proved statistically significant ($p$ <0.05 for both groups).

*Endotoxin and gut microbiota changes*

The present study in the gut microbiota composition showed that, in comparison with isolated NAFLD cases, both CAD and AH groups developed a marked tendency

towards changes in the concentration of *Bacteroidetes* and *Firmicutes,* however, without reaching any statistical significance. On the other hand, a statistically significant drop in the *Actinobacteria* concentration was discovered only in CAD group (by 41.16%, $p$ <0.05) whereas AH patients displayed an insignificant decrease by 12.70%.

Thus, anthropometric disorders, proinflammatory abnormalities, and changes in the gut microbiota composition were established in NAFLD patients with concomitant CVD, all of which require particular research.

NAFLD patients, i.e. with concomitant CAD, displayed a significant increase in the level of ET, comparing with the control, while ET concentration was significantly higher in comorbid pathology. In NAFLD patients the ET level correlated with the relative content of *Firmicutes* (r = 0.39, $p$ <0.05) and their ratio to *Bacteroidetes* (r = 0.29, $p$ <0.05) and had an inverse relation with the relative content of *Bacteroidetes* (r = –0.42, $p$ <0.01), and was also significantly ($p$ <0.05) higher in women than in men and progressively increased with age (r = 0.30, $p$ <0.05). NAFLD patients with concomitant CAD displayed an inverse dependence of the ET level on the relative content of *Bacteroidetes* (r = –0.42, $p$ <0.01) and a direct dependence on their ratio to *Firmicutes* (r = 0.29, $p$ <0.05) which progressively increased with age (r = 0.30, $p$ <0.05).

Quantitative changes in the intestinal microbiota in NAFLD patients were characterized by a decrease in *Lactobacillus* spp. in 85.00% of patients and *Bifidobacterium spp.* in 31.67%, *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*) in 88.00%, *Akkermansia muciniphila* (*A. muciniphila*) in 81.67% and *Faecalibacterium prausnitzii* (*F. prausnitzii*) in 33.33% against the background growth of gram-negative flora, i.e. *Enterobacter* spp./*Citrobacter* spp. in 45.00%, *E. coli* in 18.33% and *B. fragilis* group in 30.00%. At the same time, the levels of *Bifidobacterium* spp. (r = 0.37, $p$ <0.05), *Bacteroides fragilis* group (*B. fragilis* group) (r = –0.43, $p$ <0.01), *Escherichia coli* (*E. coli*) (r = –0.41, $p$ < 0.01) and total bacterial mass (r = –0.39, $p$ <0.05) correlated with the level of ET.

NAFLD patients with concomitant CAD showed similar changes in the intestinal microbiota, but they were present in a larger number of patients. Thus, a decrease in the content of *Lactobacillus* spp. was identified in 83.33% of patients, *Bifidobacterium* spp. in 29.17%, *B. thetaiotaomicron* in 87.50%, *A. muciniphila* in 79.17% and *F. prausnitzii* in 33.33%, while there was also an increase in the gram-negative flora, i.e. *Enterobacter* spp./*Citrobacter* spp. in 45.83% of patients, *B. fragilis* group in 29.17%, *E. coli* in 20.83%. The level of ET had an inverse relation with the content of *B. fragilis* group (r = –0.43, $p$ <0.01), *E. coli* (r = –0.41, $p$ <0.01) and total bacterial mass (r = –0.39, $p$ <0.05).

While divided into two groups depending on *B. fragilis* group abundance, among NAFLD patients there were five times more subjects found being with an increased *E. coli* (44.44% vs 8.33%, respectively) and almost three times more subjects with an increase in the *B. fragilis* group / *F. prausnitzii* ratio (94.44% vs 34.50%, respectively) and twice as many subjects with anaerobic imbalance in the gut microbiota (83.33% vs 37.50%, respectively). Among patients with a decreased level of *A. muciniphila* there were almost one and a half times more subjects with an increased *Enterobacteriacea,* compared with among those without *A. muciniphila* deficiency (47.37% vs 20.00%, respectively). Patients with increased levels of *Enterobacter* spp./*Citrobacter* spp. displayed the increased *E. coli* content five times more often than patients with the normal range of *Enterobacter* spp./*Citrobacter* spp. (36.36% vs 7.69%, respectively). In turn, among subjects with an increase in *E. coli* there were more patients with a significant increase in *Enterobacteriaceae* (81.3% vs 36.8%), the *B. fragilis* group (68.8 % vs 20.6%) and anaerobic imbalance in the gut microbiota (75.0% vs 44.1%).

NAFLD patients with concomitant CAD showed an association of an increase in *B. fragilis* group (compared with the patients with its normal values) with a significant increase in *E. coli* (45.83% vs 8.33%) and the anaerobic imbalance in the gut microbiota (83.33% vs 38.33%). Patients with a decreased level of *A. muciniphila* (compared with subjects with its normal values) displayed an increase in the *Enterobacteriaceae* (44.8% vs 29.4%).

## Discussion

The aim of this study was to investigate changes in the gut microbiota composition in NAFLD and CVD (comorbid in most cases). As it is known, intestinal microorganisms form a community, the gut microbiota, a diverse ecosystem within the human body, which combines about $10^{14}$ microorganisms.[10] The vast majority of gut bacteria fall in the main five phylotypes, namely *Bacteroidetes* (56%), *Firmicutes* (29%), *Actinobacteria* (6%) and *Proteobacteria* (4%).[10] Disturbances in the qualitative and quantitative composition of gut microbiota contribute to the development of various conditions, including NAFLD[21] and CVD.[22] The nature of the pathogenetic links between gut microbiota changes in CVD in NAFLD patients still remains the subject of scientific research.

Atherosclerosis is the key pathogenetic mechanism of CVD evolvement and progression.[23] Among the factors of atherogenesis, gut microorganisms are believed to play an important if not the leading role.[24–26] Cui et al. discovered changes in the quantitative composition of the gut microbiota at the phylotype level, e.g. a decrease in *Bacteroidetes* with a reciprocal increase in *Firmicutes.*[27] Despite the unfavorable results of the meta-analysis of the antibiotic effectiveness in CAD patients,[28] recent evidence demonstrated that the gut microbiota plays a causal role in atherosclerosis due to intestinal barrier dysfunction, endotoxemia, proinflammatory changes, the synthesis of trimethylamine N-oxide, bile acids and microbial metabolites,[29] as well as adipose tissue dysfunction, disorders in lipid[30] and carbohydrate metabolism.[31] In addition, microorganisms can realize their influence through the synthesis of short-chain fatty acids, butyrate in particular – the fermentation end products of dietary fiber, which are the main source of energy for colonocytes that support the intestinal mucosal barrier.[32]

However, the results of our study did not identify significant changes in *Bacteroidetes* and *Firmicutes,* despite certain visible tendencies. On the contrary, the results of this study found a statistically significant decrease in the *Actinobacteria* concentration. The study of open sources,

however, shows that *Actinobacteria* mostly remain beyond scientific research.

Contrary to the preliminary data on the increased amount of *Actinobacteria* in the structure of atherosclerotic plaque,[33] the results of the Tampere Sudden Death Study did not confirm this but showed that despite the tendency towards reduction in *Bifidobacterium* spp., the main representative of *Actinobacteria*, in CAD patients there was an inverse relation between the quantity of these bacteria and the severity of fibrosis, which suggests a favorable role of *Bifidobacterium* spp. in the prevention of coronary atherosclerosis.[34] Potential mechanisms that account for these effects include the ability of *Bifidobacteria* to improve the structural and functional state of the intestine, which leads to inhibition of bacterial translocation and endotoxinemia reduction.[35]

Changes in lipid metabolism is another phenomenon associated with gut microbiota. In particular, after 12 weeks of high fat diet in ApoE-/- mice, Li et al. observed a significant increase in the of quantity of *Firmicutes* with a decrease in *Actinobacteria, Bacteroidetes,* and *Verrucomicrobia*,[36] and negative correlation between the phylotype of *Actinobacteria* and the index of hyperlipidemia, weight gain, relative amount of epididymal fat and liver weight.[36]

Other clinical studies showed that the quantity of *Actinobacteria* depends on physical activity. In particular, endurance exercises and/or cardiac exercise significantly increase the concentration of these microorganisms in the intestinal content.[37] Conversely, a sedentary lifestyle is a risk factor for both NAFLD and CAD.[38,39]

We can declare, NAFLD patients with concomitant CAD displayed an association of an increase in *B. fragilis* group (in contrast to patients with its normal ranges) with a significant increase in *E. coli* (44.44% and 8.33%, respectively) as well as an anaerobic imbalance in gut microbiota (83.33% and 37.50%, respectively). Patients with a decreased level of *A. muciniphila* (in contrast to those who did not have it) showed an increase in *Enterobacteriaceae* (47.37% and 20.00%, respectively). Similar to patients with isolated NAFLD, subjects with concomitant CAD showed a mutually potentiating relation between increased levels of *Enterobacter* spp./*Citrobacter* spp. and an increase in *E. coli* and *B. fragilis* group in connection to *E. coli*., which were more prominent in group with comorbid course of NAFLD with CAD.

Against the background of the above changes, both groups displayed an increase in gram-negative flora: *Gamma-proteobacteria* and bacteria of the *B. fragilis* group, which explains endotoxinemia, since these bacteria are the source of ET. Conversely, both groups showed the inverse correlation of ET concentration with the level of *Bacteroidetes*, *E. coli* and the total bacterial mass, which confirms the assumption that a decrease in the number of the above microorganisms is associated with their death and, accordingly, subsequent destruction, which leads to the release of an excess amount of lipopolysaccharide from their cells and endotoxinemia. The observed changes in the intestinal microbiota, i.e. an increase in *Bacteroides*, *Proteobacteria*, *E. coli* against the background of a decrease in *A. muciniphila,* agreed with the data of other studies.[40]

A detailed analysis of the intestinal microbiome revealed that the overwhelming majority of patients in both groups displayed an increase in *B. fragilis* spp. and, accordingly, an increase in the *B. fragilis* group / *F. prausnitzii* ratio, which is worth noting given their known triggering effect on the formation and progression of inflammatory processes, while a decrease in *F. prausnitzii*, which realizes anti-inflammatory activity. Enrichment of the gut microbiota with gram-negative flora (*Bacteroides fragilis* group, *Enterobacter* spp./*Citrobacter* spp., *E. coli*) had a mutually potentiating character and often occurred against the background of a decrease in the content of *A. muciniphila*. At the same time, NAFLD patients with concomitant CAD, these changes were encountered in a larger number of patients. Taking into account the literature data, which show the relation between an increase in *Bacteroidetes* and *Proteobacteria* with the risk of type 2 diabetes mellitus and chronic endotoxinemia, as well as an increase in *Bacteroidetes* with systemic low-grade inflammation,[40] the results of this study emphasize its particular relevance.

## Conclusion

NAFLD patients with concomitant CVD, developed significant violations of anthropometric parameters and pro-inflammatory markers. There were no noticeable changes in liver function, lipid and carbohydrate metabolism with all groups of patients, which can be explained through the appropriate therapeutic measures.

The research in the gut microbiota composition assessed by via the real-time quantitative polymerase chain reaction showed statistically significant changes in patients with NAFLD and concomitant CAD, i.e. a decrease in the concentration of *Actinobacteria* by 41.37% ($p$ 0.05), compared with the patients with isolated NAFLD. However, the dynamics of the other phylotypes, *Bacteroidetes* and *Firmicutes,* were only tendencies.

There was a "metabolic" type of endotoxinemia found in NAFLD patients, i.e. in patients with concomitant CAD, which had age-related features. The revealed changes in the intestinal microbiota were associated with the level of endotoxinemia, which confirms their role in an increase in the concentration of ET due to an increase in gram-negative flora, which is a source of ET, as well as due to a decrease in *F. prausnitzii* and *A. muciniphila*, which contribute to an increase in permeability of intestinal wall. At the same time, NAFLD with concomitant CAD was associated with higher levels of endotoxinemia and more prominent changes in the gut microbiota. These changes can be caused by malnutrition and reduced physical activity. All these suggest the potential role of the gut microbiota in modulating CAD, and the development of novel effective technologies, targeting specific bacteria at the microbiome liver-heart interface, i.e. antibiotics, pro-, eu- and symbiotic, which justifies further research.

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