THE COMPOSITION OF GUT MICROBIOTA IN PATIENTS WITH HYPERLIPIDEMIA

K.B. Kvit

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Key words: gut microbiota, hyperlipidemia, bacteroidetes, firmicutes, actinobacteria.

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Materials and methods. 105 patients with hyperlipidemia and 52 controls were included into the study. The examination involved the biochemical parameters, ultrasound data, elastography, identification of gut microbial composition by real-time PCR. Patients of both groups were matched by age, BMI and metabolic characteristics.

Results. There was significant difference between composition of microbiota in patients of both groups – Actinobacteria and F/B index were significantly higher in patients with hyperlipidemia. There was negative correlational relationship between Bacteroidetes and Firmicutes and Firmicutes/Bacteroidetes ratio in patients with hyperlipidemia (r=-0.74, r=-0.69 respectively). Furthermore, there was strong positive correlation between Firmicutes and CRP (r=0.6) and apo B (r=0.8). Meanwhile, Actinobacteria in patients with hyperlipidemia had the positive correlation within triglycerides (r=0.67) and cholesterol (r=0.51).

Conclusions. The composition of the intestinal microbiota is different between group of patients with hyperlipidemia and controls. The decreasing of Bacteroidetes leads to Firmicutes growth, that influences on CRP and apo B level increasing in patients with hyperlipidemia. The increasing of Actinobacteria in patients with hyperlipidemia leads to cholesterol and triglycerides level growth.

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composition. Sequencing of 16S ribosomal RNA genes of amplified bacterial nucleic acids got from feces or biopsy examples of the intestinal mucosa, permitted to re-identify and classify representatives of the intestinal microflora. It has been presented that the adult human gastrointestinal tract contains around 1012 microorganisms in 1 ml of substance, which are represented by approximately 1000 species. Also, the investigations have demonstrated that this number can be a lot bigger – no less than 1,800 genera and 15,000-36,000 species of bacteria [3].

The dominant classes in human intestine are Bacteroidetes and Firmicutes, they constitute more than 90% of all bacteria. Actinobacteria as one of the basic phylum of intestinal microbiota are similarly imperative part of bacteria. Human studies have demonstrated that a decreasing of Bacteroidetes and increasing of Firmicutes are directly connected with obesity and type 2 diabetes. Also, a decrease of Bifidobacterium was found in patients with overweight, obesity and type 2 diabetes. Additionally, a decrease of Bifidobacterium was found in patients with overweight, obesity or type 2 diabetes. Interestingly, the level of Bifidobacterium correlated with anti-inflammatory effect, according to some data [4,5,6].

Currently, the studies, dedicated to the microbiota composition in patients with hyperlipidemia, are insufficient. Hence, there is an interest in exploring the hyperlipidemia as the result of the syndrome, that includes bacterial violation, with profound analysis of preventive and aggressive factors that impact on cholesterol level changes.

The aim of study

To explore the composition of the intestinal microbiota in patients with hyperlipidemia (including its different types), as well as the potential connection of biochemical markers with different types of bacteria.

Research materials and methods

105 patients with hyperlipidemia (64 men and 41 women) aged from 32 to 64 years (average age 52.17±2.09 years) were examined in «Medicover Ukraine» (Lviv, Ukraine), «Agency «Truskavetsk squirto» (Truskavets, Ukraine), Lviv, Lviv Emergency Hospital (Lviv, Ukraine) during 2017-2020 years. 52 control subjects (28 men and 24 women) aged from 30 to 56 years (an average 48.25±2.47 years) were matched with main group by age and metabolic characteristics. All control subjects had normal lipid range and no history of coronary disease. The average waist circumference in the main group was 98.6±1.09 cm (in men), 86.3±0.65 cm (in women), while in controls – 91.2±2.1 cm in men, 82.7±1.8 in women.

The exclusion criteria for both groups were – diabetes mellitus, viral hepatitis, autoimmune hepatitis, acute coronary syndrome in last 10 years. None of both groups subjects was taking drugs known to affect lipid profile or microbiota composition, including antibiotic medicines 1 month before and during the data. All patients have got the recommendations for diet, that required to avoid the Western diet products and high cholesterol containing products, with the aim to exclude the impact of exogenous cholesterol on the microbiota composition, during the study.

Biochemical tests. Both groups of patients underwent biochemical evaluation of serum that included blood cell count, lipid profile (total cholesterol (TC), high-density lipoproteins (HDL), low-density lipoproteins (LDL)), very low-density lipoproteins (VLDL), triglycerides (TG), C-reactive protein (CRP), alaninaminotransferase (ALT), aspartaminotransferase (AST), gamma-glutamyl transpeptidase (GGTP), bilirubin (total, direct, indirect), apolipoprotein B (apo B), apolipoprotein A1 (apo A1). Biochemical tests were carried out using commercially available test kits.

Ultrasound examination of liver was proved to all patients of both groups with the aim to diagnose the fatty liver infiltration as one of the additional factor, that influences on hyperlipidemia occurrence. The ultrasound signs for fatty infiltration included a diffuse increase in the echogenicity of the liver parenchyma, decreased attenuation on the liver and the ratio between the brightness level of the liver and the right kidney, that was calculated for the hepato-renal index (HRI) determination [7]. Also, the diagnosis of fatty liver infiltration was based on liver transient elastography. According to this data, patients with fatty liver disease characteristics from both groups were excluded from the study.

Sample collection and DNA extraction. Fresh stool samples were provided by each subject in a stool container on site. Within 10 min upon defection, the fecal sample was aliquoted and aliquots were immediately stored at 20 °C for 1 week until DNA isolation. DNA was extracted from 1.5-2 frozen stool aliquots using the phenol-chloroform method by protocol. DNA was finally eluted in 200 μl elution buffer. The DNA quantity and quality was measured by NanoDrop ND-8000 (Thermo Scientific, USA). Samples with a DNA concentration less than 20 ng or an A 260/280 less than 1.8 were subjected to ethanol precipitation to concentrate or further purified, respectively, to meet the quality standards.

Oligonucleotide primers. Quantification of different taxa by qPCR using primers targeting the 16S rRNA gene, specific for Firmicutes, Actinobacteria and Bacteroidetes, as well as universal primers was performed. The primer sequences were: Bacteroidetes: 978cbF AAACCTAAAKGGAATTGACGG (Forward) and cfb967R GTGAGGTTTCCCTCGGCTAT (Reverse); Firmicutes: 928F–fim GAAACCTAAAGGAATTGACG (Forward) and 1040FIRM R ACCATGCACCACTCTGTC (Reverse); Actinobacteria: Act920F3 TAMCCCGCAAGGCTTA (Forward) and Actl200R TCCTCCCACTCTCGCC (Reverse) and universal bacterial 16S RNA sequences: 926F AAACCTAAAKGGAATTGACG (Forward) and 1062R CTCACCRACGCGACGTAG (Reverse).

PCR amplification. PCR reaction was performed in real-time thermal cycler Rotor-Gene 6000 (QIAGEN, Germany). The PCR reaction conditions consisted of an initial denaturing step of 5 min at 95 °C, 30 cycles of 95 °C for 15 s, annealing for 15 s and 72 °C for 30 s, and a final elongation step at 72 °C for 5 min. Every PCR reaction contained 0.05 units/μl of Taq polymerase (Sigma Aldrich), 0.2 mM of each dNTP, 0.4 μM of each primer, 1× buffer, ~10 ng of DNA and water to 25 μl. Samples were amplified with all primer pairs in triplicates. The Ct (univ and spec) were the threshold cycles registered by the thermocycler. The average Ct...
value obtained from each pair was transformed into percentage with the formula.

Identification of microbial composition. Determination of microbial composition at the level of major microbial phyla was carried out by identification of total bacterial DNA, and DNA of Bacteroidetes, Firmicutes and Actinobacteria was performed with quantitative real-time PCR (qRT-PCR), using gene-targeted primers.

Statistical analysis was carried out using Statistica 11.0. Statistical analysis of the independent variables was done using the Student’s t-test (two-tailed) for unpaired data. Associations between biochemical parameters, bacteria phyla and serum lipoproteins in the whole group, were examined by calculating Pearson’s product moment correlation coefficients (r) on raw data. Between-group differences for normally distributed data were compared using an independent sample t-test, with correction for multiple parameter testing. Differences with p-values ≤ 0.05 were considered to be statistically significant. The results are expressed as means ± s.e.m.

The results and their discussion

Patient characteristics are shown in Table 1.

<table>
<thead>
<tr>
<th>Variable (normal range)</th>
<th>Main group (105)</th>
<th>Control group (52)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>43.03±2.67</td>
<td>37.5±2.6</td>
<td>≥0.05</td>
</tr>
<tr>
<td>BMI, kg/m2</td>
<td>26.15±0.67</td>
<td>24.2±1.21</td>
<td>≥0.05</td>
</tr>
<tr>
<td>Glucose, mm/l</td>
<td>5.4±0.28</td>
<td>5.1±0.12</td>
<td>≥0.05</td>
</tr>
<tr>
<td>Apo B, g/l (normal range 0.66-1.33 men, 0.6-1.17 women)</td>
<td>1.25±0.05</td>
<td>0.84±0.03</td>
<td>≥0.05</td>
</tr>
<tr>
<td>Apo A1, g/l (normal range 1.04-2.02 men, 1.08-2.25 women)</td>
<td>1.47±0.04</td>
<td>1.5±0.03</td>
<td>≥0.05</td>
</tr>
<tr>
<td>Bilirubin total, mmol/l (normal range &lt;21)</td>
<td>12.9±1.44</td>
<td>11.1±1.16</td>
<td>≥0.05</td>
</tr>
<tr>
<td>Direct bilirubin, mmol/l (normal range ≤5)</td>
<td>3.1±0.28</td>
<td>2.6±0.31</td>
<td>≥0.05</td>
</tr>
<tr>
<td>Indirect bilirubin, mmol/ (normal range &lt;75% of bilirubin total)</td>
<td>10.4±1.28</td>
<td>8.4±1.16</td>
<td>≤0.05</td>
</tr>
<tr>
<td>AST, IU/L (normal range &lt;40)</td>
<td>28.9±4.5</td>
<td>22.7±1.8</td>
<td>≤0.05</td>
</tr>
<tr>
<td>ALT, IU/L (normal range &lt;41)</td>
<td>42.1±8.4</td>
<td>26.7±3.8</td>
<td>≤0.05</td>
</tr>
<tr>
<td>AST/ALT ratio (normal range 0.91-1.75)</td>
<td>0.87±0.08</td>
<td>0.98±0.05</td>
<td>≤0.05</td>
</tr>
<tr>
<td>GGTP, IU/L (normal range &lt;55 men, &lt;38 women)</td>
<td>49.2±10.7</td>
<td>27.5±4.95</td>
<td>≤0.05</td>
</tr>
<tr>
<td>CRP, mg/l (normal range ≤5)</td>
<td>3.31±0.6</td>
<td>2.17±0.31</td>
<td>≤0.05</td>
</tr>
<tr>
<td>Cholesterol, mmol/l (normal range ≤5.2)</td>
<td>6.3±0.26</td>
<td>4.45±0.13</td>
<td>≤0.05</td>
</tr>
<tr>
<td>Triglycerides, mmol/l (normal range ≤1.7)</td>
<td>1.77±0.29</td>
<td>1.1±0.14</td>
<td>≤0.05</td>
</tr>
<tr>
<td>LDL, mmol/l (normal range ≤2.59)</td>
<td>4.03±0.23</td>
<td>2.34±0.08</td>
<td>≤0.05</td>
</tr>
<tr>
<td>VLDL, mmol/l (normal range ≤0.26-1.0)</td>
<td>0.94±0.13</td>
<td>0.58±0.07</td>
<td>≤0.05</td>
</tr>
<tr>
<td>HDL, mmol/l (normal range ≥1.56)</td>
<td>1.4±0.08</td>
<td>1.56±0.08</td>
<td>≥0.05</td>
</tr>
</tbody>
</table>

According to the Table 1, patients of main and control groups were well matched for age and BMI. Besides, the level of apo B and ApoA1 in both groups was not significantly different (≥0.05). The bilirubin level was almost equal for patients of the main and control groups, except indirect bilirubin, that was higher in patients with hyperlipidemia.

The significant difference was matched between AST, ALT, GGTP and CRP level between comparative groups, where these markers were higher in patients with hyperlipidemia. Furthermore, the level of almost all lipids components (TC, LDL, VLDL, TG) was reliably higher in patients of the main group (≥0.05), except the HDL cholesterol, which rate was slightly lower than in controls.

As well as groups were relevant, the microbiota composition was evaluated, where the main phylum of bacteria was explored – Bacteroidetes, Firmicutes, Actinobacteria. Similarly, the Firmicutes/Bacteroidetes ratio was used. The results of analysis are presented in the Table 2.

| The composition of intestinal microbiota in patients with hyperlipidemia and in controls |
|-----------------------------------------------|-----------------|-----------------|-----|
| Variable (normal range) | Main (n=105) | Control (n=52) | p   |
| Bacteroidetes (%) (normal range 15-40) | 40.70±25.43 | 52.98±13.62 | ≤0.05|
| Firmicutes (%) (normal range 20-60) | 39.43±23.77 | 33.85±12.47 | ≥0.05|
| Actinobacteria (%) (normal range 10-30) | 8.95±9.41 | 5.36±1.76 | ≤0.05|
| Firmicutes/Bacteroidetes ratio (normal range 1-5) | 2.98±1.61 | 0.75±0.54 | ≤0.05|

In view of the Table 2, the composition of microbiota was significantly different between group of patients with hyperlipidemia and controls – the proportion of Actinobacteria, Firmicutes/Bacteroidetes ratio was higher in hyperlipidemia group, Bacteroidetes was higher in controls.

Taking into account the results, we have processed the correlational relationship between different phylum of bacteria and biochemical markers in patients with hyperlipidemia.

Firstly, the positive correlation between total cholesterol and LDL, CRP and apo B was fixed in patients with hyperlipidemia (r=0.9, r=0.7, r=0.96 respectively). The positive strong relationship between LDL and CRP (r=0.7), direct (r=0.55), indirect (r=0.61) bilirubin and apo B (r=0.9) was marked. Also, the positive correlation was fixed among TG and VLDL (r=0.98) and TG and apo B (r=0.6). The biggest number of correlational relationships was associated with CRP and apo B. CRP, except the previously marked correlations, had the strong
association with ALT (r=0.74), AST (r=0.8) and GGTP (r=0.99). Apo B was linked with total (r=0.96), indirect (r=0.96), direct bilirubin (r=0.98) and CRP (r=0.79).

Remarkably, there was found strong negative correlation between Bacteroidetes and Firmicutes (r=-0.74), Bacteroides and Firmicutes/Bacteroidetes index (r=-0.69) in patients of main group. Moreover, there was strong positive correlation among Firmicutes and CRP (r=0.6) and apo B (r=0.8). Actinobacteria in patients with hyperlipidemia had the positive correlation within triglycerides (r=0.67) and cholesterol (r=0.51).

Published data, dedicated to gut microbiota in patients with hyperlipidemia, are limited and controversial. Considering the current evidence, the main attention is paid to the system of impact the gut microorganisms to the metabolic health, including the lipid profile [8,9]. On instance, the data, where authors emphasized the way of influence through the short chain fatty acids (SCFAs). In vitro studies demonstrated that propionate reduces cholesterol rate by decreasing the enzyme activity of hepatic 3-hydroxy-3-methylglutaric-CoA synthase (HMGC) and 3-hydroxy-3-methylglutaric-CoA reductase (HMGC). Likewise, in vivo data using H2O as a tracer demonstrated that cholesterol synthesis rate was diminished in rodent livers by dietary propionate supplementation [10,11].

The role of acetate in cholesterol homeostasis has got less consideration, however Fushimi et al. suggested that serum cholesterol levels are influenced by acetate. The mRNA dimension of cholesterol 7a-hydroxylase was increased in the liver upon acetate supplementation. In accordance with this perception, acetate supplementation diminished hypercholesterolemia in humans. The Bacteroidetes phylum mostly produces acetate and propionate, while the Firmicutes phylum has butyrate as its essential metabolic finished result. Most bacterial movement happens in the proximal colon where substrate accessibility is highest. Close to the distal colon, the accessibility of substrate decreases and the extraction of free water reduces diffusion of substrates and microbial products. This makes the proximal part of the colon the foremost site of fermentation. Especially, nondigestible carbohydrates are fermented in the proximal colon by saccharolytic microorganisms, primarily by such essential fermenters as Bacteroidetes [12,13].

We did not investigate the level of SCFAs in patients of the main and control group, taking into account another design of study. On the other hand, we have examined the composition of microbiota, including bacteria’s phyla—Bacteroidetes, Firmicutes and Actinobacteria, that are closely connected with SCFAs. There was significant higher level of Actinobacteria and Bacteroidetes in hyperlipidemia group.

Based on the literature evidence, obesity is often accompanied by Firmicutes phylum, that increases in patients with BMI higher than 30 and correlates with visceral adiposity. Conversely, the Bacteroidetes is named as the preventers of fat deposition [14].

In the course of the study, the correlational relationship between different biochemical markers and gut bacteria phylum was examined.

In summary, there was negative correlation between Bacteroidetes and Firmicutes in patients with hyperlipidemia. The decreasing of Bacteroidetes was provoking the Firmicutes growth. Moreover, the Firmicutes rise influenced on the CRP and apo B ratio increasing. There is well-known fact about the direct impact of apo B and CRP on the atherosclerosis occurrence. Based on this, the possibility of Firmicutes to rise up the level of CRP and apo B in patients with hyperlipidemia is worsening risk factor and must be known in care of patients with lipids dysregulation (Fig. 1).

![Fig. 1. The correlational relationship between Bacteroidetes, Firmicutes and biochemical markers in patients with hyperlipidemia.](image)

Fig. 1. The correlational relationship between Bacteroidetes, Firmicutes and biochemical markers in patients with hyperlipidemia.

Noticeably, there was positive correlation between Actinobacteria and triglycerides and cholesterol (r=0.48) (Fig. 2). This fact is one more point to consider that Actinobacteria could be the important factor in patients with hyperlipidemia because the increasing of lipids could be the first step for atherosclerosis development.

![Fig. 2. The correlational relationship between Actinobacteria and biochemical markers in patients with hyperlipidemia.](image)

Fig. 2. The correlational relationship between Actinobacteria and biochemical markers in patients with hyperlipidemia.

The result, that are demonstrated in our study are quaintly contradictory. Simultaneously, this data could be explained by profound understanding the possible impact of gut microbiota on metabolic balance, among the lipids rate.

According to the data, along with the liver, intestinal lint cells are an important source of endogenous cholesterol. Microorganisms of the digestive tract significantly affect the rate of restoration of the intestinal epithelium, and,
subsequently, additionally control the arrangement of endogenous cholesterol. The dimension of cholesterol in serum relies upon the severity of its absorption from the digestive tract [15]. The absorption is identified with the transit rate of neutral sterols through the digestive system, the concentration of ions in the intestine, the nearness and level of affinity of intestinal receptors for lipoproteins or microorganisms associated with the transformation of cholesterol [16]. Likewise, numerous intestinal microorganisms effectively deconjugate bile acids. Free bile acids reduce cholesterol absorption from the digestion tracts. Contingent upon the quantitative substance in the lumen of volatile fatty acids formed by bacteria during anaerobic digestion of carbohydrates and fats, the absorption of calcium, magnesium and zinc cations differs generally, which in the indirect way influences the rate of cholesterol in the blood [17].

Not surprisingly, that different studies in humans tried to connect the association of the gut microbiome and lipid levels correlating with circulating TG, LDL, TC, and HDL levels [18]. Unfortunately, the majority of these studies have compared type 2 diabetic (T2D) or obese individuals with healthy controls. They have consistently reported a negative correlation between TG levels and gut microbiome diversity, a general measure of gut health. Concomitantly, there are some opposite data, where the Firmicutes are connected with lower lipids rate. For example, circulating TG levels in obese and T2D individuals were found to associate with lower abundance of Clostridium species (Firmicutes phylum), independently of body mass index (BMI). Negative correlations with gut microbiome diversity were also reported for LDL. On the other hand, HDL cholesterol was found to be positively associated with microbial richness and with abundance of Clostridium species (Firmicutes phylum) [19].

A recent study by Fu and coworkers examined for the first time the association between the gut microbial composition and lipids in the general population. They assessed gut microbial composition in 893 individuals from the LifeLines cohorts using 16s rRNA sequencing. The gut microbiome explained 6% of the variation in triglycerides and 4% of the variation in HDL at the population level. Gut microbiome diversity was found to correlate negatively with TG levels and positively with HDL levels in line with several reports linking increased diversity to more favorable cardiometabolic outcomes. They also identified several taxa to be associated with TG and HDL independently of BMI. These include Eggerthella (Actinobacteria Phylum), Pasteurellaceae (Proteobacteria Phylum), and Butyricimonas (Bacteroidetes Phylum). One of the most cited microbiome factors in obesity is the shift in the ratio of the Firmicutes and Bacteroidetes phyla. Reversal of this ratio has not yet been demonstrated, and whether it can alter cholesterol levels in humans remains disputed [20,21].

With regards to our study, there was difference between balance of bacteria phylum in patients with hyperlipidemia in comparison with patients with normal lipid range. Additionally, there was strong correlational relationship, that showed the tendency of influence the Bacteroidetes phylum decreasing with Firmicutes and Firmicutes/Bacteroidetes ratio increasing in patients with hyperlipidemia. Moreover, this factor influenced on the CRP and apo B level growth. Both of these markers are connected with future atherosclerosis development [22]. Our next studies will be in continuing of this work with profound analysis of microbiota in patients with hyperlipidemia. Moreover, our future interest will be paid to including the further factors that could impact on progress and occurrence of hyperlipidemia – genetic, environmental factors and liver and intestine status. Especially, the main attention will be dedicated to possible preventive impact of different bacteria phyla on the pro-atherosclerosis biochemical markers growth in patients with hyperlipidemia.

**Conclusions**

1. The composition of gut microbiota in patients with hyperlipidemia is significantly different from controls.
2. The composition of microbiota is different in group with hyperlipidemia and controls – Actinobacteria and F/B index are significantly higher in patients with hyperlipidemia.
3. The decreasing of Bacteroidetes leads to Firmicutes increasing, that influences on CRP and apo B level growth in patients with hyperlipidemia.
4. Actinobacteria increasing could provoke triglycerides and cholesterol level growth.

**Список літератури**

References


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