Gut microbiota changes and novel markers associated with liver steatosis in obese patients

Abstract. Background. Liver steatosis is a common condition that can progress to steatohepatitis, fibrosis, and cirrhosis and increases the risk of death from cardiovascular and liver complications. Understanding the link between steatosis and non-alcoholic fatty liver disease, obesity, and gut microbiota is essential. Recent studies have revealed that gut microbiota plays a crucial role in developing this condition, highlighting the importance of microbiota control. The purpose of the study was to detect changes in gut microbiota and new markers associated with hepatic steatosis in obese patients.

Materials and methods. The study involved 60 men aged 38 to 65, divided into two groups: 32 patients with hepatic steatosis (experimental group) and 28 with no steatosis (controls). As part of the study, the levels of the lipogram were determined, anthropometric measurements were made, a bioimpedance analysis of the body was performed, as well as liver ultrasound and shear wave elastography. The gut microbiota of all participants was also examined using sequencing technologies (material collected from stool samples).

Results. In the experimental group, there are significantly more patients with overweight, dyslipidemia (hypercholesterolemia, triglyceridemia, high low-density lipoproteins, high atherogenicity coefficient, and low high-density lipoproteins). Also, patients with hepatic steatosis are more likely to have an excessive percentage of fat and an excessive amount of visceral fat, hepatomegaly due to the craniocaudal size of the liver, and increased liver stiffness. Regarding the intestinal microbiota, there is an increase in bacterial groups belonging to the Bacteroidetes. Our analysis showed that specific markers such as body mass index, blood lipid profile, body fat percentage, and liver ultrasound parameters are essential for diagnosing steatosis. Body mass index above 24.9 kg/m² and increased waist circumference were associated with steatosis. Bioimpedance analysis parameters, including body fat percentage and relative visceral fat level, were also crucial indicators. Dyslipidemia, with increased levels of total cholesterol, triglycerides, low-density lipoproteins, high atherogenicity coefficient, and lower high-density lipoproteins, was related to steatosis. The liver stiffness was significantly higher among patients with steatosis, indicating additional risk of liver fibrosis. Shear wave elastography can be a valuable tool for detecting liver fibrosis.

Conclusions. Patients with steatosis were characterized by signs of obesity (increased waist circumference, body mass index) and dyslipidemia, higher percentage of adipose tissue, relative amount of visceral fat, craniocaudal liver size, liver stiffness, and low levels of high-density lipoproteins. An increase in the gut microbiota of bacterial groups belonging to the Bacteroidetes has been observed.

Keywords: obesity; liver steatosis; intestinal microbiota
Introduction

Liver steatosis represents a widespread condition that is a part of the general pathological process, which may lead to steatohepatitis under certain conditions and subsequently to liver fibrosis and even cirrhosis [1, 2]. It should be emphasized that patients with liver steatosis are at an increased risk of death from both cardiovascular diseases and liver-related complications [3].

The pathogenetic role of steatosis in liver damage is recognized regardless of its origin. However, researchers are particularly interested in detecting steatosis in the context of non-alcoholic fatty liver disease due to its widespread occurrence and close association with metabolic disorders [4]. This is especially crucial as steatosis is highly prevalent among individuals with obesity, diabetes, and dyslipidemia [5]. Additionally, it is known that fatty liver degeneration can occur long before the development of metabolic syndrome. In contrast, liver steatosis may develop even with a slight increase in body weight, as in this case, redistribution of lipid content in tissues and metabolic disorders can occur [6].

Experimental studies have revealed that the bacteria living in our intestines have an equally important role in the development of metabolic dysfunction-associated fatty liver disease (MAFLD) [7]. Lifestyle changes and increased physical activity can alter the state of our liver through their influence on the gut microbiota composition. It is worth noting that the detected changes in the composition of the gut microbiota in patients with MAFLD can be managed by the use of particular drugs, such as probiotics, prebiotics, or synbiotics, allowing to improve the condition of patients with MAFLD significantly [8].

The timely detection and quantification of both the state of the liver and the gut microbiota have become crucial objectives for hepatology, endocrinology, and other areas of the healthcare system overall, given the high prevalence of steatosis.

The purpose of the study was to identify gut microbiota changes and novel markers associated with liver steatosis in obese patients.

Materials and methods

Sixty men aged 38 to 65 years (average of 48.6 ± 10.8 years) were involved in the study. Non-inclusion criteria were involved in the study. Non-inclusion criteria were the presence of alcoholic, drug-induced, viral, autoimmune liver damage, and storage diseases.

All patients were examined for the presence of fatty hepatitis according to the European Association for the Study of the Liver — European Association for the Study of Diabetes — European Association for the Study of Obesity Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease [1] using liver ultrasound in the absence of apparent reasons for the secondary build-up of fat in the liver (alcohol abuse, use of hepatotoxic drugs, viral hepatitis infection, autoimmune and hereditary diseases).

Exclusion of alcoholic liver damage was performed based on reports of daily alcohol consumption in a dose of at least 50.0 g of pure ethanol for 25 years and screening for alcohol abuse (CAGE questionnaire — Cut down, Annoyed, Guilty, Eye-opener). Hepatitis B virus etiology was identified using serological markers of hepatitis B virus (HBsAg determination), hepatitis C virus — serological markers of hepatitis C virus (anti-HCV), as well as information regarding acute viral hepatitis in the previous medical records. Autoimmune reactions were evaluated based on the serological markers of autoimmune liver damage.

Two groups were formed depending on the presence of steatosis: experimental (EG) — 32 people with ultrasound-confirmed steatosis, and control (CG) — 28 patients without steatosis.

Biochemical blood serum parameters were studied using an automatic analyzer, Beckman Coulter AU680 (USA), which detected the levels of total cholesterol, triglycerides, high-density lipoproteins (HDL), and low-density lipoproteins (LDL). The atherogenicity coefficient (AC) greater than 3 was considered to be elevated.

Bioimpedance analysis was conducted using Tanita BC-587 body composition analyzer scales (Tanita, Japan) to determine patients' body weight, body mass index (BMI) (kg/m²), bone mass (kg), skeletal muscle mass (kg), water content (%), body fat percentage (%), visceral (internal) fat level (units), basic metabolism (kcal), biological age (calculated based on the body’s metabolic rate) (years). We used the following indicators for the study: body fat percentage and visceral fat level. The upper limits of the normal reference range accepted during bioimpedance analysis in the studied age group were as follows: for the body fat — 20 % and less, visceral (internal) fat levels — 12 and less, whereas BMI — 24.9 kg/m² and less. Patients’ waist circumference (WC) was also measured as the primary criterion for metabolic disorders. WC exceeding 102 cm was considered elevated.

A liver ultrasound examination was conducted using a Toshiba (Canon) Apio 500 Platinum device with a 2–5 MHz convex probe under standard conditions: in the morning, on an empty stomach, in a horizontal position lying on the back. All patients underwent standard ultrasound to identify functional and structural changes in the internal organs; liver dimensions (mm), outlines, structure, and echogenicity were assessed.

Shear wave elastography was performed on a Toshiba (Canon) Apio 500 Platinum device in 2D diagnostic mode using a CA1-7A probe. The measurements were taken in real-time at a depth of 20–60 mm from the capsule to determine the average indicators that characterize the stiffness of the liver parenchyma (kPa). Threshold values were used to assess the stage of fibrosis: F0 — no fibrosis (up to 5.8 kPa); F1 — mild fibrosis (5.9–7.2 kPa); F2 — moderate fibrosis (7.3–9.5 kPa); F3 — advanced fibrosis (9.6–12.5 kPa); F4 — liver cirrhosis (> 12.5 kPa) according to the METAVIR scale.

The gut microbiota was evaluated by determining the fecal bacterial composition using a polymerase chain reaction-based sequencing method in a certified laboratory DIA-GEN, which provided results in the form of metagenomic analysis. The most used method for this purpose is 16S rRNA gene sequencing. This technique targets a specific region of the bacterial 16S rRNA gene, allowing for the identification and classification of bacterial species present in the sample collected from the stool.

The results were processed in Microsoft Excel using descriptive statistics, Student’s t-test, and z-criterion to compare two variables. The statistical reliability of the markers
was evaluated using the SPSS software based on contingency tables, and indicators of diagnostic value were calculated. The correlation between steatosis and the indicator studied was considered confirmed by a module if the association coefficient exceeded 0.5 (or 0.3 for the contingency coefficient). The results were presented as M ± m, where M is the arithmetic mean, m is the mean square deviation; n is the number of patients examined in the group. The difference was considered statistically significant if p < 0.05.

Results

Patients of the EG had significantly higher levels of total cholesterol compared to the CG (6.5 ± 1.4 mmol/l vs. 4.0 ± 0.6 mmol/l). In addition, significantly higher triglycerides (2.3 ± 0.8 mmol/l vs. 1.1 ± 0.1 mmol/l), LDL, and AC were observed in the group with liver steatosis. The mean value of HDL was not significantly different in both groups of patients. The results are shown in Table 1.

According to the information presented in Table 2, WC in patients with hepatic steatosis was significantly greater than in the CG (109.0 ± 12.0 cm vs. 97.6 ± 9.8 cm). BMI was also significantly higher in the group with steatosis compared to the CG patients (28.0 ± 2.7 kg/m² vs. 21.6 ± 1.7 kg/m²), as was the body fat percentage and the relative visceral fat level (32.3 ± 6.1 % vs. 20.9 ± 4.2 % and 9.4 ± 3.3 units vs. 4.0 ± 1.5 units, respectively).

The liver ultrasound results (Table 3) indicate a significantly greater craniocaudal (right) liver lobe length in patients with steatosis compared to the CG (160.5 ± 11.0 mm vs. 130.4 ± 13.4 mm) and a significantly higher index of liver stiffness in the EG compared to the CG (5.5 ± 0.6 kPa vs. 3.8 ± 1.3 kPa). The left liver dimension was not significantly different between the groups of patients (60.3 ± 5.6 mm vs. 57.1 ± 6.5 mm).

We obtained the following results for each group after comparing the number of patients with indicators exceeding the reference range. The number of patients with increased WC was higher in the EG, but it was not significant (19/32 vs. 11/28). However, a significantly more patients in the EG had high BMI (28/32 vs. 1/28), high levels of total cholesterol (27/32 vs. 1/28), triglycerides (24/32 vs. 6/28), high AC (30/32 vs. 8/28), high body fat percentage (31/32 vs. 8/28). There were significantly more patients with an insufficient HDL level in the EG compared to CG (29/32 vs. 15/28). Higher levels of LDL compared to the reference range (14/32), higher than normal relative visceral fat level

Table 1. Blood lipid profile

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EG, n = 32</th>
<th>CG, n = 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>6.5 ± 1.4*</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>2.3 ± 0.8*</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>4.6 ± 1.2*</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.1 ± 0.2</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>AC, units</td>
<td>5.2 ± 1.8*</td>
<td>2.7 ± 1.0</td>
</tr>
</tbody>
</table>

Note: here and in Tables 2, 3: * — p < 0.05 compared to CG values.

Table 2. Results of anthropometry and bioimpedance analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EG, n = 32</th>
<th>CG, n = 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>WC, cm</td>
<td>109.0 ± 12.0*</td>
<td>97.6 ± 9.8</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.0 ± 2.7*</td>
<td>21.6 ± 1.7</td>
</tr>
<tr>
<td>Body fat percentage, %</td>
<td>32.3 ± 6.1*</td>
<td>20.9 ± 4.2</td>
</tr>
<tr>
<td>Visceral fat level, units</td>
<td>9.4 ± 3.3*</td>
<td>4.0 ± 1.5</td>
</tr>
</tbody>
</table>

Table 3. Liver ultrasound and shear wave elastography

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EG, n = 32</th>
<th>CG, n = 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Craniocaudal (right) lobe length, mm</td>
<td>160.5 ± 11.0*</td>
<td>130.4 ± 13.4</td>
</tr>
<tr>
<td>Left liver size, mm</td>
<td>60.3 ± 5.6</td>
<td>57.1 ± 6.5</td>
</tr>
<tr>
<td>Liver stiffness index, E, kPa</td>
<td>5.5 ± 0.6*</td>
<td>3.8 ± 1.3</td>
</tr>
</tbody>
</table>

Table 4. Analysis of the diagnostic value of additional screening markers of the liver steatosis risk

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Accuracy, %</th>
<th>Association/contingency coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>WC</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
<td>0.38</td>
</tr>
<tr>
<td>BMI</td>
<td>87.5</td>
<td>95.0</td>
<td>90.0</td>
<td>0.98*</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>82.5</td>
<td>95.0</td>
<td>86.7</td>
<td>0.98*</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>75.0</td>
<td>80.0</td>
<td>76.7</td>
<td>0.85*</td>
</tr>
<tr>
<td>LDL</td>
<td>42.5</td>
<td>100.0</td>
<td>61.7</td>
<td>0.44*</td>
</tr>
<tr>
<td>HDL</td>
<td>90.0</td>
<td>45.0</td>
<td>75.0</td>
<td>0.76*</td>
</tr>
<tr>
<td>AC</td>
<td>92.5</td>
<td>70.0</td>
<td>85.0</td>
<td>0.93*</td>
</tr>
<tr>
<td>Body fat percentage</td>
<td>97.5</td>
<td>70.0</td>
<td>88.3</td>
<td>0.98*</td>
</tr>
<tr>
<td>Visceral fat level</td>
<td>27.5</td>
<td>100.0</td>
<td>51.7</td>
<td>0.33*</td>
</tr>
<tr>
<td>Craniocaudal (right) lobe length</td>
<td>72.5</td>
<td>100.0</td>
<td>81.7</td>
<td>0.68*</td>
</tr>
<tr>
<td>Left liver size</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Liver stiffness</td>
<td>32.5</td>
<td>90.0</td>
<td>51.7</td>
<td>0.63*</td>
</tr>
</tbody>
</table>

Note: * — statistically confirmed correlation between the marker and the risk of liver steatosis.
(9/32), and increased craniocaudal size of the liver were only observed among patients of the EG (23/32). All the above indicators were within the reference range in controls. The left liver size was not increased in patients of both groups.

The following research phase is statistical analysis, which calculates the chances of accurately diagnosing liver steatosis using individual indicators — markers. Indicators of diagnostic value, such as sensitivity, specificity, and accuracy, were employed to determine the reliability of the diagnosis. Based on them, the odds ratio was defined — a number that shows how much the absence or presence of a particular result is associated with the presence or absence of a specific disease in a statistical group, and the association (or contingency) coefficient, which characterizes how close is the stochastic association between qualitative signs — alternative random variables. The sensitivity, specificity, accuracy, and association (or contingency) coefficient are shown in Table 4.

Based on the obtained data, WC, although it is a marker of metabolic disorder, did not indicate the presence of liver steatosis. However, all other assessed indicators can be considered risk markers of liver steatosis. Namely, BMI with a sensitivity of 87.5 %, specificity of 95.0 %, and accuracy of 90.0 % indicates the presence of liver steatosis. Blood lipid indicators are also of high importance: total cholesterol with a sensitivity of 82.5 %, specificity of 95.0 %, and accuracy of 86.7 % indicates the presence of steatosis, as well as triglycerides with a sensitivity of 75.0 %, specificity of 80.0 %, accuracy of 76.7 %; LDL with a sensitivity of 42.5 %, specificity of 100.0 %, accuracy of 61.7 %; HDL with a sensitivity of 90.0 %, specificity of 45.0 %, accuracy of 75.0 %; AC with a sensitivity of 92.5 %, specificity of 70.0 %, and accuracy of 85.0 %. The indicators of bioimpedance analysis, specifically the percentage of body fat tissue and the relative visceral fat level, are equally important. Excess visceral fat level with a sensitivity of 97.5 %, specificity of 70.0 %, and accuracy of 88.3 %, and relative visceral fat level with a sensitivity of 27.5 %, specificity of 100.0 % and accuracy of 51.7 % indicate liver steatosis. Regarding the liver ultrasound results, the craniocaudal size of the liver indicated hepatic steatosis with a sensitivity of 72.5 %, specificity of 100.0 %, and accuracy of 81.7 %. In contrast, the liver stiffness index had a sensitivity of 90.0 %, specificity of 45.0 %, accuracy of 61.7 %; HDL with a sensitivity of 82.5 %, specificity of 95.0 %, and accuracy of 90.0 %, accuracy of 90.0 % indicates the presence of liver steatosis. However, all other assessed indicators can be considered risk markers of liver steatosis. Our choice was not accidental, as it is known that non-alcoholic fatty liver disease and liver steatosis, which is one of its components, are pathogenetically closely linked [9, 10]. This topic is of paramount importance since there is an ongoing discussion among scientists worldwide regarding redefining the term “non-alcoholic fatty liver disease” to “metabolically associated liver disease”, thereby transferring the disease from the rank of diseases of exclusion to a nosology with clearly defined diagnostic criteria [11].

It’s not just the scientific aspect that makes this topic relevant. As of 2015, around 604 million people across the globe are suffering from obesity [12]; the prevalence of type 2 diabetes and hypertension also contributes to the development of liver steatosis, with a risk of transforming into steatohepatitis and later into liver cirrhosis and, in some cases, hepatocellular carcinoma [13]. All of this underscores the urge for early detection of liver steatosis.

Our statistical analysis has confirmed the importance of specific markers such as BMI greater than 24.9 kg/m², blood lipid indicators, body fat percentage, and relative visceral fat level, along with specific liver ultrasound parameters (craniocaudal liver size and liver stiffness index).

In addition to the significant practical value, the data we obtained provide a specific understanding of the “risk group” — patients most likely to develop steatosis. Our results emphasize that despite increased WC is an important marker of metabolic disorder, it did not become the factor that determined the presence of hepatic steatosis. In contrast to WC, BMI > 24.9 kg/m² was reliably associated with steatosis. Bioimpedance analysis parameters (body fat percentage and relative visceral fat level) were crucial. The above data highlights the importance of conducting a comprehensive examination rather than focusing on the visible deposition of fatty tissue in certain areas.

Blood lipid indicators, such as increased levels of total cholesterol, triglycerides, LDL, high AC, and lower HDL, are directly pathogenetically related to steatosis. This indicates that in dyslipidemia, the patient should undergo a comprehensive examination with screening for liver steatosis.
Regarding the gut microbiota, the predominance of \textit{Bacteroidetes} over all groups of bacteria is noteworthy. An interesting feature was the predominance of this type of bacteria in patients with hepatic steatosis compared to other groups and across the total population combined. The identified changes raise many concerns, namely, whether such a redistribution of the gut microbiota composition is a consequence of the deterioration of the metabolic status of the liver and obesity, or possibly the opposite, an extreme increase in bacteria of the \textit{Bacteroidetes} type is capable of triggering liver steatosis and obesity [14, 15].

The significance of liver stiffness in liver ultrasound cannot be overstated, as it was significantly higher among patients with steatosis. Additionally, exceeding the threshold values was often observed among participants with steatosis compared to those without it, which indicates additional risks of liver fibrosis in such patients [16–18]. The routine performance of shear wave elastography can serve as a valuable tool in determining liver steatosis.

**Conclusions**

Patients diagnosed with hepatic steatosis had significantly higher WC and BMI, as well as higher levels of total cholesterol, triglycerides, LDL, and AC, higher body fat percentage and relative visceral fat level, significantly larger cranio-caudal (right) liver size, and higher liver stiffness when compared to those without this condition. However, the mean value of HDL did not differ significantly between the two groups.

In the group with hepatic steatosis, there were significantly more patients with increased BMI, high levels of total cholesterol, triglycerides, LDL, AC, and low HDL. They also had an increase in the following parameters: body fat percentage and relative visceral fat level, cranio-caudal liver size, and liver stiffness. Furthermore, the growth of \textit{Bacteroidetes} groups was observed in the gut microbiota.

Markers associated with the presence of hepatic steatosis include high BMI, total cholesterol, triglycerides, LDL, AC, bioimpedance analysis indicators, namely the body fat percentage and the relative visceral fat level, certain parameters of the liver ultrasound, such as cranio-caudal liver size and liver stiffness index, and low HDL levels.

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2

1 Львівський національний медичний університет імені Данила Галицького, м. Львів, Україна
2 КНП ЛОР «Львівська обласна клінічна лікарня», м. Львів, Україна

Зміни мікробіоти кишечника та нові маркери, пов’язані зі стеатозом печінки, у пацієнтів з ожирінням

Резюме. Актуальність. Стеатоз печінки є поширенним ураженням, яке може прогресувати до стеатогепатиту, фіброзу та цирозу, і збільшує ризик смерті від серцево-судинних та церевних ускладнень. Важливою особливістю стеатозу є бактеріальна мікробіота кишечника. У недавніх дослідженнях з’ясували, що кишкова мікробіота є ключовим фактором у формуванні стеатозу печінки. Особливо вагомою є призначення мікробіоти кишечника в утворенні інфекційних та неінфекційних кишкових дисфункцій, які впливають на розвиток стеатозу печінки.

Мета: Систематизацію змін мікробіоти кишечника і бактеріальних груп, що належать до типу Bacteroidetes, у пацієнтів з стеатозом печінки.

Матеріали та методи. Для використання в результативному аналізі було використано 60 параозових осіб віком від 30 до 65 років, яких розділили на дві групи: 32 пацієнти зі стеатозом (експериментальна група) та 28 осіб без стеатозу (контрольна група). У рамках дослідження визначено рівні ліпопротеїнів низької щільності та високої щільності, а також зв’язок з електрометричним розміром печінки. Ці показники були досить високими у пацієнтів зі стеатозом печінки.

Висновки. В експериментальній групі було виявлено значно більше осіб із надмірною масою тіла, порівняно з контрольною групою. Високі рівні ліпопротеїнів низької щільності та низької щільності вірогідно вищі серед пацієнтів з стеатозом печінки.

Ключові слова: кишкова мікробіота, стеатоз печінки, Bacteroidetes, мікробіотометрия.