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Small intestinal bacterial overgrowth and non-alcoholic fatty liver disease diagnosed by transient elastography and liver biopsy

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Abstract

Background: We aimed to determine if there was a higher incidence of small intestinal bacterial overgrowth (SIBO) in non-alcoholic fatty liver disease (NAFLD) than in patients without NAFLD. Moreover, we assessed whether patients with significant fibrosis (SF) had a higher incidence of SIBO compared with patients with non-significant or no liver fibrosis.

Methods: NAFLD was diagnosed in 117 patients by using Fibroscan with a controlled attenuation parameter (CAP) as well as liver biopsy (LB). SIBO was defined by esophagogastroduodenoscopy with an aspiration of the descending duodenum.

Results: Patients with non-alcoholic steatohepatitis (NASH) and those with SF on LB had a significantly higher incidence of SIBO than patients without NASH and those without SF, respectively ($P < .05$). According to histological characteristics, there was a higher proportion of patients in the SIBO group with higher steatosis and fibrosis grade, lobular and portal inflammation, and ballooning grade ($P < .001$). In multivariate analysis, significant predictors associated with SF and NASH were type 2 diabetes mellitus (T2DM) and SIBO. Moreover, in multivariate analysis, significant predictors that were independently associated with SIBO were T2DM, fibrosis stage and ballooning grade (OR 8.80 (2.07-37.37), 2.50 (1.16-5.37) and 27.6 (6.41-119), respectively). The most commonly isolated were gram-negative bacteria, predominantly *Escherichia coli* and *Klebsiella pneumoniae*.

Conclusion: In this relatively large population of patients, we used a gold standard for both SIBO (quantitative culture of duodenum's descending part aspirate) and NAFLD (LB), and we demonstrated that NASH patients and those with SF had a higher incidence of SIBO. Moreover, significant predictors independently associated with SIBO were T2DM, fibrosis stage and ballooning grade. Although TE is a well-investigated method for steatosis and fibrosis detection, in our study, independent predictors of

SIBO were histological characteristics of NAFLD, while elastographic parameters did not reach statistical significance.

1 | INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a chronic parenchymal liver disease closely related to metabolic syndrome (MetS) and its components: obesity, diabetes mellitus type 2 (T2DM), hypertension and dyslipidaemia. Nowadays, it is the leading chronic liver disease (CLD) and the leading cause of liver enzyme alteration. Furthermore, it is set to become the leading indication for liver transplantation by 2030. One of the essential characteristics of NAFLD is liver parenchyma steatosis appearing in the absence of alcohol abuse or at least in amounts harmful to the liver. The amount of consumed alcohol considered harmless for men and women is <30 and <20 g/day, respectively. When considering pathophysiology, insulin resistance (IR) is the central player behind NAFLD development.[1-4] In recent years, the role of gut microbiota draws the attention of many authors in the context of the CLD. Over 100 years ago, B. Hoefert was the first to emphasise the significance of changes in the gut microbiota composition of CLD.[5,6]

There is growing evidence suggesting that gut bacteria modify host metabolism and predispose them to MetS and further respective consequences. Keeping in mind that NAFLD is closely related to MetS and IR, recent studies indicate the role of gut microbes in NAFLD development.[6-8]

Small intestinal bacterial overgrowth (SIBO) syndrome is defined as excessive bacteria in the small intestine as well as changes in intestinal bacteria type. Most authors imply SIBO diagnosis as $\geq 10^5$ of bacteria per 1 mL of aspiration from proximal jejunum. One of SIBO's characteristics is gram-negative bacteria excess found in the proximal small bowel, which can induce hepatic steatosis, as shown in animal models. Also, several studies have indicated a relationship between SIBO and NAFLD with bacterial endotoxins and tumour necrosis factor (TNF) as effective mediators.[6,10] In most studies, non-invasive methods were used to establish a diagnosis of NAFLD (liver enzymes and ultrasonography) or SIBO (D-xylose, glucose and lactulose hydrogen breath tests).[5-13] However, liver biopsy (LB) remains the gold standard for NAFLD diagnosis as well as the identification of its necroinflammatory form (ie, non-alcoholic steatohepatitis; NASH) and liver fibrosis.[1-5]

Moreover, the golden standard for establishing SIBO diagnosis is esophagogastroduodenoscopy (EGD) with an aspiration of the descending duodenum or jejunum.[5-13] As mentioned previously, studies evaluating the frequency and risk factors for SIBO in NAFLD patients are non-existent or lacking, especially ones implementing a liver biopsy and EGD in the diagnostic process. Consequently, we aimed to determine whether patients with NAFLD (defined by elastography and histology) had a higher incidence of SIBO (defined by EGD with the aspiration of the descending duodenum) compared with patients without NAFLD. Furthermore, we investigated whether patients with significant fibrosis had a higher incidence of SIBO than patients with non-significant or no liver fibrosis.

What's known

Several studies have indicated a relationship between SIBO and NAFLD with bacterial endotoxins and tumour necrosis factor (TNF) as effective mediators. In most studies, non-invasive methods were used to establish a diagnosis of NAFLD (liver enzymes and ultrasonography) or SIBO (D-xylose, glucose and lactulose hydrogen breath tests). However, liver biopsy remains the gold standard for the diagnosis of NAFLD and its necroinflammatory form (ie non-alcoholic steatohepatitis; NASH) and liver fibrosis. Moreover, the golden standard for establishing SIBO diagnosis is esophagogastroduodenoscopy (EGD) with an aspiration of the descending duodenum or jejunum.

What's new

We have found patients with NASH and those with significant liver fibrosis to have a higher incidence of SIBO diagnosed by EGD with an aspiration of the descending duodenum, a gold standard for SIBO diagnosis. Moreover, SIBO patients have a higher incidence of all histological characteristics of NAFLD, steatosis, inflammation, NAS score and significant fibrosis. Independent predictors of NASH were T2DM, glucose, HbA1c and SIBO, while independent predictors of significant fibrosis were T2DM and SIBO. In multivariate analysis, significant predictors independently associated with SIBO were T2DM, fibrosis stage and ballooning grade. Although TE is a well-investigated method for steatosis and fibrosis detection, in our study, independent predictors of SIBO were histological characteristics of NAFLD. At the same time, elastographic parameters did not reach statistical significance.

2 | METHODS

2.1 | Study participants and design

This observational cross-sectional study was conducted between January 2018 and March 2019 at the Clinical Hospital Center Rijeka (CHC Rijeka). Based on the presence of abnormal liver enzymes and/or an ultrasound scan showing an echobright liver, NAFLD was suspected in patients with one or more MetS components. We used

transient elastography (TE) (FibroScan) with controlled attenuation parameter (CAP) and liver stiffness measurements (LSM) as well as liver histology to establish NAFLD diagnosis. This study was approved by UHC Rijeka Ethics Committee. Written informed consent before participation in this study was given by all patients. The study was conducted in agreement with the International Conference on Harmonisation guidelines on Good Clinical Practice and under the declaration of Helsinki. All authors had access to the study data, reviewed and approved the final manuscript.

2.2 | Main analyses

The primary aim was to determine whether patients with NASH, defined by liver histology and those with NAFLD activity score (NAS) ≥5, had a higher incidence of SIBO than patients without NASH. Also, we investigated whether patients with significant fibrosis (F2-F4 by Metavir) had a higher incidence of SIBO compared with patients without significant liver fibrosis (F0-F1 by Metavir).

The secondary aim was to analyse the difference in clinical, laboratory, elastographic and histological characteristics in patients with and without SIBO and its predictors.

2.3 | Inclusion and exclusion criteria

Inclusion criteria counted patients older than 18 years who were negative for hepatitis B and C virus and could have an LB and EGD. Also, patients had to be able to give written informed consent. Patients with alcohol consumption above recommended limits, defined as more than 14 drinks per week in women and more than 21 drinks per week in men over 2 years, were excluded from the study. Patients who used antibiotics and probiotics in the last 3 months were not a part of this study.

Moreover, exclusion criteria included patients with an active malignancy, pregnant women, patients with ascites, cardiac failure and/or significant valvular disease, patients with other metabolic, autoimmune liver disease, hepatotoxic medications intake, patients refusing LB or EGD, or ones participating in another clinical trial within the preceding 30 days. Patients with invalid FibroScan measurements or those that did not have both Fibroscan and LB done were excluded from this analysis (Figure 1).

2.4 | Patient characteristics

Laboratory data, clinical and anthropometric measurements were gathered at the same time as elastographic measurements and LB. Using the International Diabetes Federation[14] definition, we defined MetS by no less than three of the following abnormalities: anti-hypertensive treatment or blood pressure ≥130/85 mm Hg; waist circumference >94 cm for men and >80 cm for women; a fasting plasma glucose level ≥5.6 mmol/L or previously diagnosed T2D or use of any hypoglycaemic drugs; triglyceride levels >1.7 mmol/L and/or HDL-cholesterol <1.29 mmol/L for women and <1.04 mmol/L for men or lipid-lowering treatment. We calculated body mass index (BMI) using the formula: weight/height^2 (kg/m^2). The laboratory parameters used in our study were: liver enzymes (aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT) and alkaline phosphatase (ALP), complete blood count, serum glucose, fasting insulin, haemoglobin A1c (HbA1c), lipid profile (including triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol), uric acid and ferritin levels. The laboratory parameters of all patients were analysed by using standard laboratory methods. Also, we calculated the homeostasis model assessment-estimated insulin resistance (HOMA-IR) score by using the following formula: HOMA-IR = [glucose (nmol/L) × insulin (μU/mL)/22.5].

2.5 | Transient elastography measurements

In order to reduce the variability of TE-measurements, all elastographic measurements were performed by a single operator.

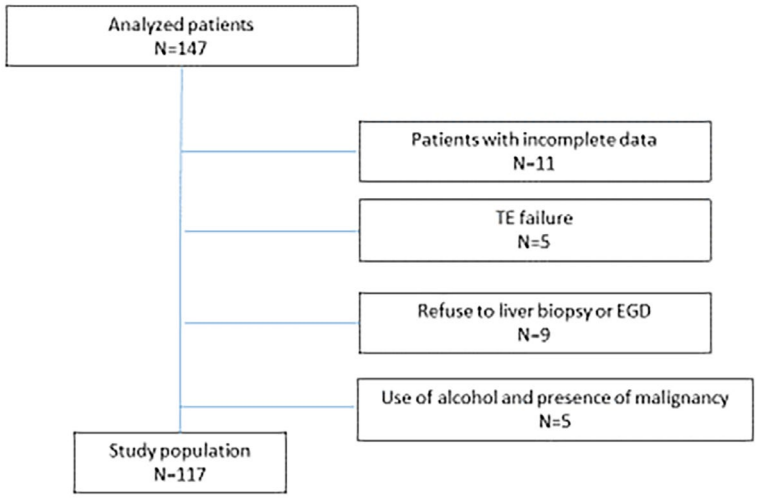

FIGURE 1 Flow chart of analyzed patients. TE, transient elastography

Fibroscan 502 Touch operating software (Echosens, Paris, France) was used. Also, we used the Medium (M+) and Large (L+) Fibroscan probe to improve accuracy. Although every patient usually requires an appropriate type of probe depending on his BMI, for this case, we used the Fibroscan 502 Touch that has an operating software with the automatic probe selection tool, which is embedded within the Fibroscan. While decibels per meter (dB/m) represented the results of elastographic parameters of liver steatosis (CAP), kilopascals (kPa) expressed the elastographic parameters of liver fibrosis. The ratio of the interquartile range (IQR) of LSM to the median (IQR/MLSM) was calculated as an indicator of variability, which means that for this analysis, we took and considered valuable only the measures with an IQR/M ratio of the LSM value <0.3, a success rate of at least 60% and at least 10 valid consecutive measurements. According to literature, significant liver fibrosis, measured as LSM, had the cut-off value of 7 kPa or more, whereas CAP value of 238 dB/m or more defined significant liver steatosis.[15]

2.6 | Liver biopsy

Percutaneous liver biopsy (LB) was performed in all analysed patients under local standard procedure. The standard procedure includes fixing the LB specimen into formalin, embedding it into paraffin, and staining it with haematoxylin, eosin and Mallory for fibrosis evaluation. Experienced pathologists blinded to the patient's clinical and FibroScan data were enrolled to analyse the slices. Using the NASH CRN scoring system, we scored steatosis (from 0 to 3), ballooning (from 0 to 2), lobular inflammation (from 0 to 3), fibrosis (from 0 to 4) and NAS.[16] NASH was defined as NAS ≥ 5.[16]

2.7 | Duodenal aspiration

Upper gastrointestinal endoscopy was done under light sedation using a gastroscope and a double lumen catheter. We collected the duodenal aspirate during EGD. The catheter assembly consisted of outer and inner tubes, the latter one being 3 cm longer. The mouth of the outer tube was blocked by an obturator. We used autoclaving for sterilising the assembly. The catheter assembly was introduced through the biopsy channel of a sterilised gastroscope reaching the descending part of the duodenum. We collected duodenal aspirate through the inner tube using a sterile syringe and used it for aerobic and anaerobic bacterial culture. Before the EGD procedure, patients had to have their usual mouth, tongue and teeth cleaning and flush mouth and gargle throat with a hexedine solution.

2.8 | Microbiological analysis

Samples were sent to the microbiology laboratory at room temperature within 3h of collection and cultured immediately for aerobic and anaerobic bacteria and yeasts. For quantitative cultures, a calibrated plastic 10-μL loop was used to plate the undiluted clinical sample. The number of colonies that appear from this 1/100th-mL sample is multiplied by 100 to give the colony-forming units per millilitre (CFU/mL) of duodenal fluid. Aerobic cultures were plated on 5% sheep blood agar, chocolate agar and MacConkey agar and incubated at 35°C for 24 hours. Anaerobic cultures on non-selective Brucella blood agar were incubated in anaerobic jars at 35°C for 48 hours. For isolation and enumeration of yeasts, chromogenic Candida agar was used and incubated for at least 48 hours. Different colonies were selected according to their morphological characteristics and purified by successive sub-culturing. Colonies from the anaerobic plates were subsequently examined for their ability to grow aerobically. Identification was based on traditional phenotypic and biochemical tests performed manually and/or by automated Vitek 2 analyser (Biomerieux, France) and appropriate, commercially available reagent cards, according to the manufacturer's instructions. Antimicrobial susceptibility testing was performed by the disk diffusion method and/or e-test following recommendations of the EUCAST.[17] SIBO was defined as a bacterial population in the small intestine exceeding 10^5 organisms/mL.

2.9 | Statistical analysis

Categorical variables are shown as percentages and continuous variables as means with a standard deviation. The distribution relationship between categorical variables values was tested using χ^2 test, if necessary, Fisher's exact test. The difference between two continuous variables was tested using a two-way independent samples t-test. We have applied false discovery rate (FDR) which uses Benjamini-Hochberg method to identify which values from data remain significant when adjusting for multiple testing. The threshold is set at 5%. The false discovery rate method (FDR = 5%) was used for multiple comparisons' correction. Multivariable logistical regression analyses were conducted to identify patient characteristics independently associated with SIBO, NASH and liver fibrosis. Univariate analysis was first performed on each variable of the independent variables to select variables for the multivariable analyses. Those factors with a *P* value <.5 in the univariate analyses were selected as candidate variables for multivariable logistical regressions. All the statistical analyses were performed using SPSS V.22.0 (SPSS Inc, Chicago, Illinois, USA). Statistical tests were two-tailed and significance was set at 0.05.

3 | RESULTS

3.1 | Characteristics of the total population

The characteristics of the total population are shown in Table 1. The mean age of the total population was 58.3 ± 11.7 years, with 47.9% of men. Type 2 diabetes mellitus, hypertension and dyslipidaemia were present in 44.4%, 75.2% and 75.3% of patients, respectively. The average BMI of the population was in the obese category (33.4 ± 5.3 kg/m^2). SIBO was present in 47.2% of patients.

TABLE 1 Characteristics of the total population

Total population	N = 117
Age (years)	58.3 ± 11.7
Gender (male), n (%)	56 (47.9)
T2DM, n (%)	52 (44.4)
Hypertension, n (%)	88 (75.2)
Dyslipidaemia, n (%)	86 (75.43)
BMI (kg/m^2)	33.4 ± 5.3
MetS, n (%)	86 (73.5)
WC (cm)	111.5 ± 13.2
HC (cm)	113.1 ± 12.6
UAC (cm)	34.1 ± 6.7
Glucose (mmol/L)	6.1 ± 1.2
HbA1c (%)	6.0 ± 1.3
HOMA-IR score	6.7 ± 4.1
Fasting insulin (mU/L)	24.3 ± 17.6
Cholesterol (mmol/L)	5.2 ± 1.3
LDL(mmol/L)	2.8 ± 1.4
HDL (mmol/L)	1.2 ± 0.4
Triglycerides (mmol/L)	1.85 ± 0.9
Urea (mmol/L)	5.7 ± 1.3
Creatinine (mmol/L)	76.6 ± 17.8
AST (IU/mL)	35.5 ± 24.1
ALT (IU/mL)	53.4 ± 28.7
GGT (IU/mL)	73.8 ± 70.7
ALP (IU/mL)	75.5 ± 24.4
Urates (mmol/L)	357 ± 73.7
SIBO, n (%)	51 (47.2)
LSM (kPa)	8.2 ± 5.2
CAP (db/m)	328.4 ± 45
Fibrosis stage	
F0, n (%)	24 (20.5)
F1, n (%)	38 (32.5)
F2, n (%)	39 (33.3)
F3, n (%)	14 (12.0)
F4, n (%)	2 (1.7)

Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; BMI, body mass index; CAP, controlled attenuation parameter; F, fibrosis; GGT, gamma-glutamyl transferase; HbA1C, haemoglobin A1c; HOMA-IR, homeostasis model assessment-insulin resistance; LSM, liver stiffness measurement; MetS, metabolic syndrome; SIBO, small intestinal bacterial overgrowth; T2DM, type 2 diabetes mellitus; UAC, upper arm circumference; WC, waist circumference; HC, waist-to-hip ratio.

3.2 | Differences in patient characteristics according to NASH and predictors of NASH

Characteristics of patients with and without NASH and with and without significant fibrosis and their comparison are shown in Table 2. Patients with NASH had a higher proportion of T2DM (55.1% vs 29.2%, $P = .021$), MetS (69.6% vs 50%, $P < .001$), SIBO (68.1% vs 29.1%, $P < .001$), BMI, ALT but lower levels of urates. In univariate analysis, significant predictors of NASH were SIBO, T2DM, MetS, glucose, HbA1c, ALT and urates. In multivariate analysis, significant predictors that were independently associated with NASH were T2DM, glucose, HbA1c and SIBO (OR 3.01 (1.5-8.5), 1.79 (1.03-3.12), 1.54 (1.12-1.91) and 5.2 (1.19-23.39), respectively (Table 3). The association between the number of bacteria and the presence of NASH is depicted in Figure 2. There was a higher number of bacteria ($P < .001$) in patients with NASH.

3.3 | Differences in patient characteristics according to liver fibrosis and predictors of liver fibrosis

Patients with significant liver fibrosis had a higher proportion of Mets (70.9% vs 53.2%) and SIBO (72.7% vs 17.7%), and higher BMI, HOMA-IR score, fasting insulin and ALT levels. In univariate analysis, significant predictors of significant fibrosis were SIBO, T2DM, BMI, HbA1c, fasting insulin, AST and ALT. In multivariate analysis, significant predictors that were independently associated with significant liver fibrosis were T2DM and SIBO (OR 1.54 (1.2-4.04), 5.58 (1.5-20.28), respectively) (Table 4). The relationship between the number of bacteria and the presence of significant fibrosis (F2-F4) is depicted in Figure 3. There was a higher difference in the number of bacteria ($P < .001$) according to the degree of fibrosis (F0 and F1 and F2 and higher).

3.4 | Differences in patient characteristics according to SIBO and predictors of SIBO

Differences in the clinical, laboratory and histological characteristics between SIBO patients and those without SIBO are showed in Table 5 and Table 6. Patients with SIBO had higher proportion of T2DM (64.7% vs 21.2%, $P < .001$), MetS (80.4% vs 57.6%, $P < .001$), BMI (35.0 ± 4.7 vs 32.2 ± 5.3 kg/m^2, $P = .013$) and NAS score (5.6 ± 1.1 vs 3.7 ± 1.5, $P < .001$). Also, according to histological characteristics, there was a higher proportion of patients in the SIBO group with higher fibrosis stage, steatosis grade, lobular inflammation, portal inflammation and ballooning grade ($P < .001$) (Table 6). In univariate analysis, significant predictors of SIBO were T2DM, BMI, glucose, HbA1c, ALT, NAS score, fibrosis stage, steatosis grade, lobular inflammation, portal inflammation and ballooning grade. In univariate analysis of SIBO predictors, CAP and LSM had a trend but did not reach statistical significance ($P = .058$, $P = .062$, respectively). In multivariate analysis, significant predictors that were independently associated with SIBO were T2DM, fibrosis stage and ballooning grade (OR 8.80 (2.07-37.37), 2.50 (1.16-5.37) and 27.6 (6.41-119), respectively) (Table 7).

3.5 | Isolated microorganisms

The most commonly isolated were gram-negative bacteria, predominantly *Escherichia coli* and *Klebsiella pneumoniae*. Among gram-positive

TABLE 2 Characteristics of patients with a and without NASH, and with and without liver fibrosis

Variables	NAS < 5 (n = 48)	NAS ≥ 5 (n = 69)	*P*	F0-1 (n = 62)	F2-4 (n = 55)	*P*
Age (years)	55.8 ± 12.7	60 ± 10.8	.156	56.4 ± 12.9	60.5 ± 10.1	.176
Gender (male), n (%)	24 (50)	32 (46.3)	.198	29 (46.8)	27 (49.1)	.953
T2DM, n (%)	14 (29.2)	38 (55.1)	.021*	21 (33.9)	31 (56.4)	.086
Hypertension, n (%)	34 (70.8)	54 (78.3)	.646	43 (69.4)	45 (81.8)	.387
Dyslipidaemia, n (%)	38 (79.2)	48 (69.6)	.646	47 (75.8)	39 (70.9)	.953
BMI (kg/m^2)	31.7 ± 4.9	34.6 ± 5.2	.015*	32.2 ± 5.5	35 ± 4.9	.017*
MetS, n (%)	24 (50)	48 (69.6)	<.001*	33 (53.2)	39 (70.9)	.159*
Glucose (mmol/L)	6.2 ± 1.3	11.8 ± 2.1	.068	6.5 ± 2.0	9.7 ± 1.7	.438
HbA1c (%)	5.8 ± 0.9	8.7 ± 1.5	.570	6 ± 1.0	9.2 ± 1.9	.387
HOMA-IR score	5.3 ± 2.9	7.5 ± 4.6	.068	5.6 ± 3.3	7.9 ± 4.6	.412*
Fasting insulin (mU/L)	19.4 ± 9.4	27.2 ± 20.8	.156	19.2 ± 10.5	30.8 ± 24.2	.438*
Cholesterol (mmol/L)	5.3 ± 1.0	5.1 ± 1.4	.719	5.0 ± 1.3	5.3 ± 1.1	.387
LDL(mmol/L)	2.8 ± 1.1	3.0 ± 0.7	.646	2.7 ± 1.0	3.0 ± 0.8	.412
HDL (mmol/L)	1.2 ± 0.3	1.3 ± 0.3	.782	1.2 ± 0.3	1.3 ± 0.4	.071
Triglycerides (mmol/L)	2.0 ± 0.9	1.9 ± 0.8	.339	1.9 ± 0.7	2.0 ± 0.9	.453
Urea (mmol/L)	5.9 ± 1.3	7.5 ± 1.6	.580	5.8 ± 1.5	8.0 ± 1.7	.438
Creatinine (mmol/L)	77 ± 17	76.3 ± 18.5	.835	78.8 ± 18.3	75.2 ± 18.4	.438
AST (IU/mL)	31.4 ± 25.5	38 ± 23	.327	30.6 ± 22.8	40.4 ± 24.8	.115
ALT (IU/mL)	41.7 ± 24.4	61.3 ± 28.9	<.001*	44.8 ± 25.4	62.7 ± 29.5	.001*
GGT (IU/mL)	71.1 ± 72.7	75.7 ± 76.5	.812	70.2 ± 69	81 ± 85.3	.438
ALP (IU/mL)	74.8 ± 22.8	76.7 ± 26.9	.791	73.7 ± 24.1	77 ± 24.4	.891
Urates (mmol/L)	374.8 ± 72.6	319.7 ± 90.3	.013*	364.3 ± 77	348.9 ± 97	.806
SIBO, n (%)	14 (29.1)	47 (68.1)	<.001*	11(17.7)	40 (72.7)	<.001*

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; F, fibrosis; GGT, gamma-glutamyl transferase; HbA1C, haemoglobin A1c; HOMA-IR, homeostasis model assessment-insulin resistance; MetS, metabolic syndrome; NAS, NAFLD activity score; SIBO, small intestinal bacterial overgrowth; T2DM, type 2 diabetes mellitus.

*P value has been corrected for multiple testing using FDR method.

bacteria, the most commonly isolated species was *Enterococcus faecalis* (Figure 4). Also, *Candida albicans* was isolated in three patients.

4 | DISCUSSION

In the last several years, there has been considerable scientific interest in associating gut microbiota with numerous diseases, including NAFLD. The anatomical and functional relationship between colon and liver ensures a theoretical basis assuming that the liver acts as a significant gut microbiota target. In the last few decades, numerous studies had been aimed at changing the gut microbiota composition in CLD patients, with preliminary results pointing towards dysbiosis having a role in liver disease progression. Furthermore, there is growing evidence showing a correlation between bacterial translocation of gut microbiota and liver steatosis.[5-11] There is increasing research interest in the relationship between SIBO, course and the severity of CLD and development of complications: ascites, encephalopathy, spontaneous bacterial peritonitis and portal hypertension.[5-12]

Gut microbiota can influence most risk factors for NAFLD development by enticing IR, increasing oxidative stress and causing changes in bile acids. In SIBO, gut barrier permeability is increased, which encourages bacterial translocation along with bacterial by-products, especially lipopolysaccharides. Research has proven an increase of endotoxins, CD-14 mRNA, nuclear factor kappa B (NF-κB) and Toll-like receptor 4 (TLR-4) in both NAFLD and SIBO. Endotoxaemia in SIBO patients likely activates TLR-4 and CD-14 receptors by stimulating NF-kB expression, which in turn increases the manufacturing of proinflammatory cytokines such as tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 6 (IL-6) and interleukin 8 (IL-8). Excessive production of these cytokines induces the development of inflammation as well as IR and is considered significant in NASH, liver fibrosis and HCC pathogenesis. Also, increased TNF-α production can be related to IR increase, a well-known trigger for the progression and development of liver fibrosis.[6-8]

Furthermore, the gut microbiota has an inhibitory effect on intestinal expression of the fasting-induced adipose factor (FIAF). This factor is an inhibitor of lipoprotein lipase (LL). Consequently, gut microbiota may increase LL activity in adipose tissue, leading to the intensification of the delivery of adipocyte-derived triglycerides and the accumulation of triacylglycerols in the liver.[6] Gut

TABLE 3 Univariate and multivariate analysis on predictors for NAS ≥ 5

NAS ≥ 5	Univariate		Multivariate	
Variables	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Age (years)	1.03 (0.99-1.07)	.061		
Gender (male), n (%)	1.32 (0.61-2.88)	.474		
T2DM, n (%)	4.41 (1.92-10.1)	<.001*	3.01 (1.5-8.5)	.021*
Hypertension, n (%)	1.35 (0.53-3.42)	.517		
Dyslipidaemia, n (%)	1.07 (0.43-2.65)	.879		
BMI (kg/m^2)	1.06 (0.99-1.14)	.086		
MetS, n (%)	2.7 (1.15-6.32)	.022*	1.18 (0.22-6.25)	.841
Glucose (mmol/L)	1.68 (1.28-2.22)	<.001*	1.79 (1.03-3.12)	.036*
HbA1c (%)	2.25 (1.38-3.68)	.001*	1.54 (1.12-1.91)	.040*
HOMA-IR score	1.073 (0.96-1.19)	.190		
Fasting insulin (mU/L)	1.01 (0.98-1.03)	.418		
Cholesterol (mmol/L)	1.03 (0.95-1.11)	.419		
LDL(mmol/L)	1.03 (0.94-1.12)	.458		
HDL (mmol/L)	1.75 (0.38-8.04)	.467		
Triglycerides (mmol/L)	1.50 (0.92-2.43)	.098		
Urea (mmol/L)	1.02 (0.96-1.08)	.386		
Creatinine (mmol/L)	0.99 (0.96-1.01)	.484		
AST (IU/mL)	1.01 (0.98-1.02)	.685		
ALT (IU/mL)	1.02 (1.01-1.03)	.024*	1.01 (0.98-1.04)	.305
GGT (IU/mL)	0.99 (0.98-1.01)	.924		
ALP (IU/mL)	0.99 (0.98-1.02)	.820		
Urates (mmol/L)	0.98 (0.97-0.99)	.004*	0.99 (0.98-1.04)	.076
SIBO, n (%)	8.75 (3.4-22.2)	<.001*	5.2 (1.19-23.39)	.030*

Note: Multivariate analysis has been adjusted for age, gender and BMI.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyl transferase; HbA1C, haemoglobin A1c; HOMA-IR, homeostasis model assessment-insulin resistance; MetS, metabolic syndrome; NAS, NAFLD activity score; SIBO, small intestinal bacterial overgrowth; T2DM, type 2 diabetes mellitus.

*$P < .05$.

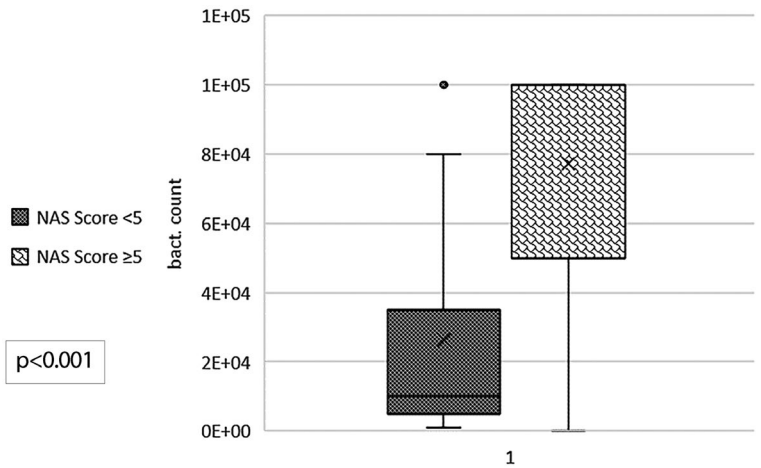

FIGURE 2 The connection between the number of bacteria and the presence of NASH. Box & whisker plot shows statistically significant difference in the number of bacteria ($P < .001$) according to NAS score <5/≥5. NAS score, nonalcoholic fatty liver disease activity index

TABLE 4 Univariate and multivariate analysis on predictors for F2-4

F2-4	Univariate		Multivariate	
Variables	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Age (years)	1.03 (0.99-1.07)	.063		
Gender (male), n (%)	1.09 (0.53-2.26)	.802		
T2DM, n (%)	2.52 (1.19-5.33)	.015*	1.54 (1.2-4.04)	.038*
Hypertension, n (%)	1.98 (0.83-4.75)	.122		
Dyslipidaemia, n (%)	0.89 (0.38-2.06)	.784		
BMI (kg/m^2)	1.12 (1.03-1.21)	.003*	1.04 (0.89-1.22)	0.574
MetS, n (%)	1.94 (0.91-4.14)	.084		
Glucose (mmol/L)	1.13 (0.95-1.33)	.155		
HbA1c (%)	1.74 (1.1-2.74)	.017*	0.98 (0.80-1.21)	.910
HOMA-IR score	1.15 (1.02-1.29)	.015		
Fasting insulin (mU/L)	1.04 (1.01-1.08)	.015*	1.04 (0.99-1.09)	.078
Cholesterol (mmol/L)	1.02 (0.96-1.08)	.497		
LDL(mmol/L)	1.02 (0.95-1.09)	.528		
HDL (mmol/L)	1.03 (0.89-1.20)	.649		
Triglycerides (mmol/L)	1.02 (0.94-1.11)	.542		
Urea (mmol/L)	1.02 (0.96-1.08)	.310		
Creatinine (mmol/L)	0.98 (0.96-1.01)	.328		
AST (IU/mL)	1.02 (1.01-1.04)	.048*	1.01 (0.98-1.02)	.578
ALT (IU/mL)	1.03 (1.01-1.04)	.002*	1.02 (0.99-1.03)	.601
GGT (IU/mL)	1.01 (0.99-1.02)	.335		
ALP (IU/mL)	1.01 (0.98-1.02)	.821		
Urates (mmol/L)	0.99 (0.98-1.01)	.709		
SIBO, n (%)	10.18 (4.17-24.8)	<.001*	5.58 (1.5-20.28)	.009*

Note: Multivariate analysis has been adjusted for age, gender and BMI.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; F, fibrosis; GGT, gamma-glutamyl transferase; HbA1C, haemoglobin A1c; HOMA-IR, homeostasis model assessment-insulin resistance; MetS, metabolic syndrome; SIBO, small intestinal bacterial overgrowth; T2DM, type 2 diabetes mellitus.

*$P < .05$.

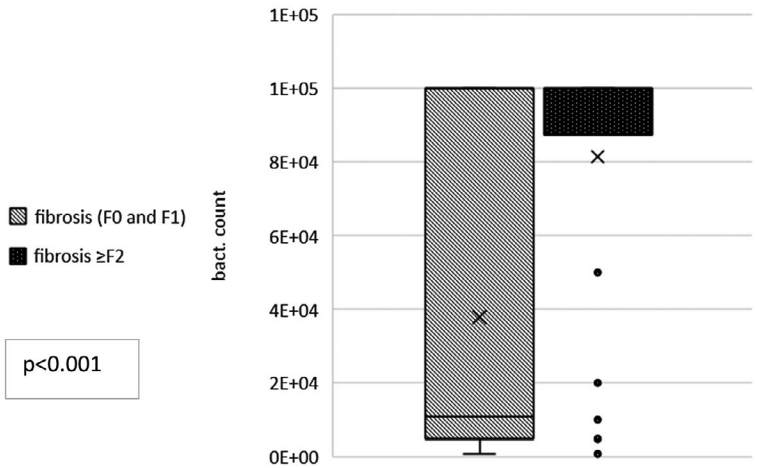

FIGURE 3 The connection between the number of bacteria and the presence of significant fibrosis (F2-F4). Box & whisker plot shows a statistically significant difference in the number of bacteria ($P = .001$) according to the degree of fibrosis (F0 and F1, and F2 and higher)

TABLE 5 Differences in clinical and laboratory characteristics between SIBO patients and those without SIBO

	SIBO (n = 51)	Non-SIBO (n = 66)	*P*
Age, years	61.0 ± 10.7	55.9 ± 12.1	.130
Gender (male), n (%)	27 (52.9)	24 (36.6)	.505
T2DM, n (%)	33 (64.7)	14 (21.2)	<.001*
Hypertension, n (%)	40 (78.4)	41 (62.1)	.750
Dyslipidaemia, n (%)	39 (76.4)	40 (60.6)	.894
BMI (kg/m^2)	35.0 ± 4.7	32.2 ± 5.3	.013*
MetS, n (%)	41 (80.4)	38 (57.6)	<.001*
HbA1c (%)	9.7 ± 1.9	5.7 ± 0.8	.130
HOMA-IR score	7.6 ± 4.6	5.6 ± 3.8	.164
Fasting insulin (mU/L)	27.2 ± 22.4	21.9 ± 12.6	.347
Cholesterol (mmol/L)	5.2 ± 1.4	5.1 ± 1.08	.949
LDL(mmol/L)	2.8 ± 1.1	2.9 ± 0.8	.655
HDL (mmol/L)	1.3 ± 0.3	1.2 ± 0.3	.775
Triglycerides (mmol/L)	2.1 ± 0.8	1.8 ± 0.9	.462
Urea (mmol/L)	8.4 ± 1.8	5.7 ± 1.3	.505
Creatinine (mmol/L)	75.9 ± 21.3	77.4 ± 14.7	.811
AST (U/L)	37.4 ± 22.8	34.3 ± 27.3	.949
ALT (U/L)	61.1 ± 24	49.3 ± 32.2	.130
ALP (U/L)	77.2 ± 21.4	76.0 ± 27.6	.505
GGT (U/L)	68.8 ± 27.6	72.2 ± 26.8	.346
CAP (db/m)	337.2 ± 41.5	320.1 ± 46.7	.156
LSM (kPa)	9.4 ± 6.2	7.4 ± 4.6	.156
NAS score	5.6 ± 1.1	3.7 ± 1.5	<.001*

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CAP, controlled attenuation parameter; GGT, gamma-glutamyl transferase; HbA1C, haemoglobin A1c; HOMA-IR, homeostasis model assessment-insulin resistance; LSM, liver stiffness measurement; MetS, metabolic syndrome; NAS, NAFLD activity score; SIBO, small intestinal bacterial overgrowth; T2DM, type 2 diabetes mellitus.
*$P < .05$.

TABLE 6 Histological characteristics in patients with and without SIBO

Histological characteristics	With SIBO (n = 51)	Without SIBO (n = 66)	*P* value
Fibrosis stage, n (%)			
F0	3 (5.9)	21 (31.8)	<.001
F1	8 (15.7)	30 (45.5)	
F2	25 (49.0)	14 (21.2)	
F3	14 (27.5)	0 (0)	
F4	1 (2.0)	1 (1.5)	
Steatosis grade			
S1	15 (29.4)	40 (60.6)	.001
S2	24 (47.1)	21 (31.8)	
S3	12 (23.5)	5 (7.6)	
Lobular inflammation			
0	0 (0)	10 (15.2)	<.001
1	9 (17.6)	47 (71.2)	
2	42 (82.4)	9 (13.6)	
Portal inflammation			
0	4 (7.8)	27 (40.9)	<.001
1	42 (82.4)	38 (57.6)	
2	5 (9.8)	1 (1.5)	
Ballooning grade			
0	0 (0)	8 (12.1)	<.001
1	5 (9.8)	38 (57.6)	
2	41 (80.4)	20 (30.3)	
3	5 (9.8)	0 (0)	
NAS score	5.6 ± 1.1	3.7 ± 1.5	<.001*

Abbreviations: NAS score, non-alcoholic fatty liver disease activity index; SIBO, small intestinal bacterial overgrowth.
*$P < .05$.

microbiota also influences bile acids metabolism. They are created in the liver, within the classical guiding cholesterol conversion into 7α-hydroxycholesterol using cholesterol 7'-hydroxylase, ultimately producing cholic and chenodeoxycholic acid (CA and CDCA respectively). Alternatively, the pathway follows the conversion of cholesterol to 27-hydroxycholesterol, facilitated by sterol 27-hydroxylase. Although it is unsure what guides a particular pathway course, the classical pathway is usually physiological, while the alternative takes place in existing liver pathology. Primary bile acids created via classical pathway are conjugated with glycine and taurine then intermittently stored in the gall bladder followed by duodenal secretion as a physiological response to food intake. Deconjugation occurs under the influence of gut microbiota when secondary bile acids are created – lithocholic and deoxycholic acid (LCA and DCA, respectively). This is followed by bile acid ileal reabsorption and return to the liver via enterohepatic circulation. The rate and quantity of reuptake depend on liver status as well as initial bile acid amounts. Farnesoid X receptor (FXR is a key player in regulation of various pathways in the body mainly through the regulation of gene expression. The FXR is usually found in the ileum and liver, where that are activated by various bile acids, including CDCA, decreasing DCA, CA and LCA affinities, respectively. This activation results in bile acid synthesis reduction and encourages liver secretion, also influencing lipid metabolism regulation and gluconeogenesis as well as regulation of various inflammatory processes in the liver.[6-8,30]

Moreover, research credits some colon bacteria, especially *E. coli*, with increased endogenous alcohol production in NASH patients. In normal conditions, with healthy colon microbiota, excess alcohol is almost immediately eliminated via alcohol dehydrogenase liver enzymes. An increased amount of alcohol and its metabolism are related to raised permeability and damage of the colon barrier, endotoxaemia, increased level of proinflammatory cytokines, and oxidative stress. This furthers the development of inflammation in the liver, which can also play a role in NAFLD pathogenesis.[6-10]

TABLE 7 Univariate and multivariate analysis on predictors of SIBO

	Univariate		Multivariate	
Variables	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Age (years)	1.03 (0.99-1.07)	.031		
Gender (male), n (%)	1.43 (0.68-2.99)	.334		
T2DM, n (%)	4.51 (2.07-9.92)	<.001*	8.80 (2.07-37.37)	.003*
Hypertension, n (%)	1.36 (0.58-3.22)	.479		
Dyslipidaemia, n (%)	1.17 (0.50-2.75)	.710		
BMI (kg/m^2)	1.11 (1.03-1.20)	.005*	1.04 (0.88-1.24)	.591
MetS, n (%)	1.73 (0.72-4.15)	.219		
Glucose (mmol/L)	1.22 (1.01-1.49)	.043*	1.04 (0.65-1.68)	.848
HbA1c (%)	2.71 (1.60-4.60)	<.001*	1.49 (0.45-4.91)	.512
HOMA-IR score	1.09 (0.98-1.22)	.082		
Fasting insulin (mU/L)	1.01 (0.99-1.04)	.180		
Cholesterol (mmol/L)	1.03 (0.89-1.20)	.614		
LDL(mmol/L)	1.02 (0.94-1.12)	.536		
HDL (mmol/L)	1.44 (0.33-6.15)	.623		
Triglycerides (mmol/L)	1.29 (0.82-2.03)	.265		
Urea (mmol/L)	1.02 (0.96-1.08)	.506		
Creatinine (mmol/L)	0.99 (0.97-1.01)	.712		
AST (IU/mL)	1.01 (0.98-1.02)	.361		
ALT (IU/mL)	1.01 (1.01-1.03)	.013*	1.03 (0.99-1.07)	.100
GGT (IU/mL)	0.98 (0.98-1.01)	.262		
ALP (IU/mL)	0.99 (0.98-1.02)	.516		
Urates (mmol/L)	0.99 (0.97-1.01)	.070		
CAP (db/m)	1.01 (0.99-1.01)	.058		
LSM (kPa)	1.08 (0.99-1.17)	.062		
NAS score	3.1 (2.01-4.78)	<.001*	1.01 (0.32-3.21)	.979
Fibrosis stage	4.42 (2.49-7.83)	<.001*	2.50 (1.16-5.37)	.018*
Steatosis grade	2.66 (1.51-4.68)	<.001*	2.61 (0.43-3.31)	.414
Lobular inflammation	25.1 (9.2-68)	<.001*	27.6 (6.41-119)	<.001*
Portal inflammation	6.72 (2.50-18)	<.001*	2.67 (0.21-33.1)	.444
Ballooning grade	16.93 (6-47.6)	<.001*	5.19 (0.64-41.6)	.120

Note: Multivariate analysis has been adjusted for age, gender and BMI.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CAP, controlled attenuation parameter; GGT, gamma-glutamyl transferase; HbA1C, haemoglobin A1c; HOMA-IR, homeostasis model assessment-insulin resistance; LSM, liver stiffness measurement; MetS, metabolic syndrome; NAS, NAFLD activity score; T2DM, type 2 diabetes mellitus.

*$P < .05$.

In our analysis, the most commonly isolated bacteria were gram-negative, with *E. coli* being one of the more commonly isolated species.

Several studies established a relationship between SIBO and MetS onset.[18-21] As per our analysis, SIBO patients had a higher incidence of MetS and its components, such as T2DM and increased BMI. Nevertheless, NASH patients with a higher incidence of SIBO also had a higher incidence of T2DM, obesity and MetS with increased HOMA-IR score when compared with NASH-free patients who, in turn, had a statistically significant decrease in SIBO incidence. Patients with significant liver fibrosis that had higher SIBO incidence also had a higher incidence of obesity defined by increased BMI levels when compared with fibrosis free or F1 fibrosis patients that had lower SIBO incidence.

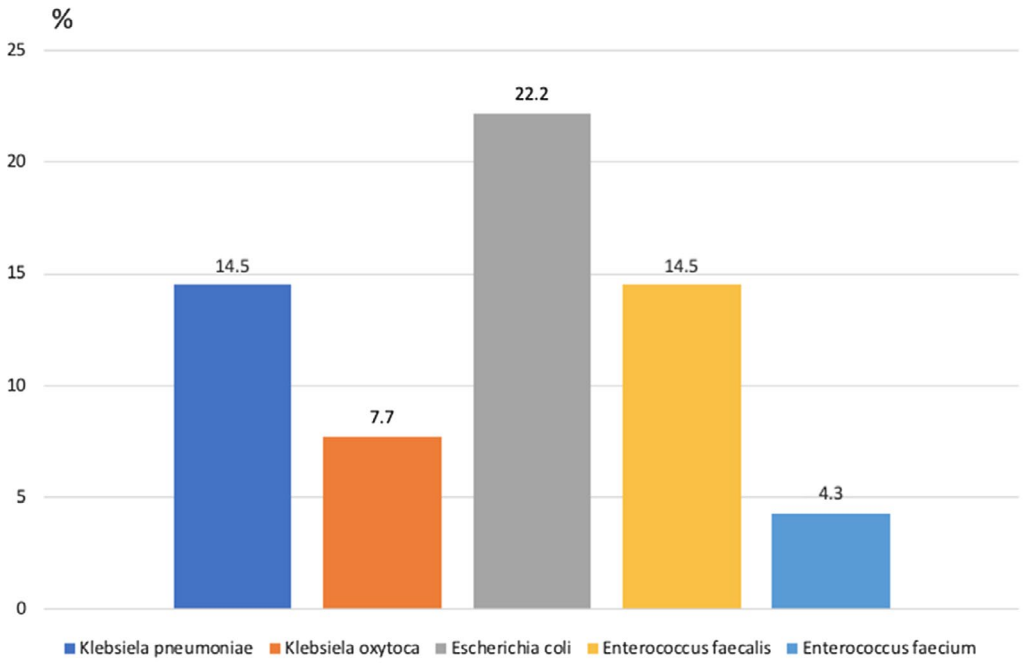

FIGURE 4 The most commonly isolated bacteria

The association between gut microbiota and metabolic disorders such as obesity, T2DM, atherosclerosis, broadly named MetS, has been becoming more apparent in the last several years.[18-20] Animal studies indicate that disturbance of gut microbiota is related to obesity.[18] Additionally, some studies suggest that gut microbiota composition in people with and without obesity is different.[19] Indeed, 10 years ago, Sabate JM et al[21] had shown that SIBO prevalence is higher in patients with obesity when compared with patients with normal BMI. Additionally, animal studies have shown that gut microbiota has a role in the deterioration of IR and T2DM and promotes metabolic endotoxaemia. Human studies confirmed that metabolic endotoxaemia also plays a role in IR and T2DM pathogenesis.[19] In our study, SIBO patients had significantly higher BMI values, which is in line with the mentioned observations.

Today we know that NAFLD arises as a consequence of MetS and its components, and NAFLD being considered a liver manifestation of MetS.[1-5] Consequently, it is not surprising that gut microbiota disturbance is related to SIBO and NAFLD onset and severity. Almost half of our patients with histologically proven NAFLD also had a proven SIBO, which correlates to previous studies.[21] SIBO and T2DM were independent predictors of the degree of NAFLD, that is, NASH and significant fibrosis. Our analysis is advantageous when considering that NAFLD was proven via LB, the gold standard for NAFLD diagnosis.

In the first part of our analysis, we have shown that patients with histologically proven NASH have a higher incidence of SIBO. Moreover, in multivariate analysis, SIBO was a significant predictor of NASH. The association between the degree of NASH and SIBO was additionally confirmed by having a higher number of bacteria ($P < .001$) in patients with NASH as well as with data showing the degree of NASH severity (as defined by NAS scoring) association to the number of bacteria found.

Also, we have shown that dysbiosis or the presence of SIBO is associated with a finding of significant liver fibrosis in NAFLD patients. Patients with significant fibrosis, as defined by F2-4 via Metavir score, have a higher incidence of SIBO. Moreover, SIBO was found to be a predictor of significant fibrosis in the multivariate analysis along with T2DM. Also, there was a difference in the number of bacteria ($P < .001$) according to the degree of fibrosis. Similar results have been published in previous studies, unlike the high prevalence of both diseases, as of yet still scarce.[10,21,22,24-26,28] We included patients only with both FibroScan and LB diagnosed NAFLD in our analysis. In a part of the previous studies, NASH was diagnosed via non-invasive methods (US abdomen, liver enzymes and transient elastography),[10,22,23] while in other studies, NASH was diagnosed solely based on LB.[21,24-26] According to recent studies, TE is a suitable method for non-invasive detection and staging of steatosis and fibrosis.[27] Although TE is a well-investigated method for steatosis and fibrosis detection, in our study, independent predictors of SIBO were histological characteristics of NAFLD. At the same time, elastographic parameters did not reach statistical significance. Actually, CAP and LSM in univariate analysis of SIBO predictors had a trend but did not reach statistical significance. Therefore, further studies regarding the role of TE in the context of SIBO are needed.

In the second part of our analysis, we have shown that patients with SIBO have a higher incidence of T2DM and MetS and a higher NAS score. When comparing all histological characteristics of NAFLD between those with SIBO and those without SIBO, there was a higher proportion of patients in the SIBO group with higher fibrosis stage, steatosis grade, lobular inflammation, portal inflammation and ballooning grade ($P < .001$) (Table 6). In multivariate analysis, significant predictors independently associated with SIBO were T2DM, fibrosis stage and ballooning grade.

SIBO screening is becoming increasingly important as research has shown that treatment has benefits on liver enzyme levels. For example, a study published 4 years ago by Gangarapu et al[29] demonstrated that the administration of antibiotics (rifaximin) has a beneficial impact on AST and GGT. Nevertheless, there is still insufficient evidence that the application of probiotics influences NAFLD treatment.[6]

In terms of SIBO diagnosis, there is substantial disagreement in data interpretation to establish which test is most appropriate. Two tests are commonly used: bacterial culture and breath tests. However, the gold standard is performing anaerobic and aerobic colony counts of small bowel luminal contents. In the context of this approach it is very important that before the EGD, patients had to have their usual mouth, tongue and teeth cleaning and had to flush mouth and gargle throat with a hexedine solution in order to avoid contamination of the culture with normal flora. On the other hand, breath testing has some advantages such as simplicity, safety and is non-invasive. Among the most frequently used are readily metabolised carbohydrates, such as glucose, lactulose, xylose, or sucrose.

Nevertheless, there are some open issues regarding breath tests. Firstly, few substrates have been investigated, but none has been identified as being superior to another. Secondly, there are evident differences in bacterial flora among patients which may determine their response to test. There are no clear recommendations regarding the optimum protocol for the administration, optimal timing and collection of breath specimens. Also, the use of antibiotics may alter the results, while the influence of PPIs is not well studied.[29,30]

In our study, we used a quantitative culture of the duodenum's descending part aspirate, the gold standard test for SIBO diagnosis. Interestingly, there are only a few studies that used the gold standard for SIBO diagnosis,[24,26,28] while other studies have used non-invasive methods such as lactose or D-xylose breath tests.[10,21-23,25]

We should emphasise that our study had some limitations. Most notably, we did not compare our results with a control group, patients without MetS, and NAFLD. Thus our results are limited by selection bias. Although it is a relative limitation because we did intend nor design this as a case-control study. Additionally, liver fibrosis is still part of a dynamic process; while we only used a cross-sectional study design, further prospective studies are needed.

We aimed to investigate the difference in SIBO incidence between patients with NASH and significant fibrosis and those NAFLD patients without NASH and significant fibrosis, which translates to those with mild liver disease and those with significant liver disease.

In this relatively large population of patients, we used a gold standard for both SIBO (quantitative culture of duodenum's descending part aspirate) and NAFLD (LB), and we demonstrated that NASH patients and those with SF had a higher incidence of SIBO. Moreover, SIBO together with T2DM was an independent predictor of degree of CLD; that is, NASH and fibrosis. SIBO patients have a higher incidence of NASH, significant fibrosis, and higher steatosis in LB. This result also showed that SIBO is associated with significant fibrosis and NASH independently of MetS and its individual components. Although TE is well investigated method for steatosis and fibrosis detection, in our study, independent predictors of SIBO were histological characteristics of NAFLD, while elastographic parameters did not reach statistical significance. Actually, CAP and LSM in univariate analysis of SIBO predictors had a trend, but did not reach statistical significance, therefore, further studies regarding the role of TE in the context of SIBO are needed.

DISCLOSURES

All authors have no conflict of interests.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

DATA AVAILABILITY STATEMENT

Data not available because of ethical restrictions.

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