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The severity of NAFLD is associated with gut dysbiosis and shift in the metabolic function of the gut microbiota

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Abstract

Background & aims—Several animal studies have emphasized the role of gut microbiota in non-alcoholic fatty liver disease (NAFLD). However, data about gut dysbiosis in human NAFLD remains scarce in the literature, especially studies including the whole spectrum of NAFLD lesions. We aimed to evaluate the association between gut dysbiosis and severe NAFLD lesions, i.e. non-alcoholic steatohepatitis (NASH) and fibrosis, in a well-characterized population of adult NAFLD.

Methods—57 patients with biopsy-proven NAFLD were enrolled. The taxonomic composition of gut microbiota was determined using 16S ribosomal RNA gene sequencing of stool samples.

Results—30 patients had F0/1 fibrosis stage at liver biopsy (10 with NASH), and 27 patients had significant F≥2 fibrosis (25 with NASH). *Bacteroides* abundance was significantly increased in NASH and F≥2 patients, whereas *Prevotella* abundance was decreased. *Ruminococcus* abundance was significantly higher in F≥2 patients. By multivariate analysis, *Bacteroides* abundance was

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independently associated with NASH and *Ruminococcus* with F≥2 fibrosis. Stratification according to the abundance of these 2 bacteria generated 3 patient subgroups with increasing severity of NAFLD lesions. Based on imputed metagenomic profiles, KEGG pathways significantly related to NASH and fibrosis F≥2 were mostly related to carbohydrate, lipid, and amino acid metabolism.

Conclusion—NAFLD severity associates with gut dysbiosis and a shift in metabolic function of the gut microbiota. We identified *Bacteroides* as independently associated with NASH and *Ruminococcus* with significant fibrosis. Thus, gut microbiota analysis adds information to classical predictors of NAFLD severity and suggests novel metabolic targets for pre/probiotics therapies.

Keywords

Non-alcoholic steatohepatitis; liver fibrosis; gut microbiome; *Bacteroides*; *Ruminococcus*

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), the liver manifestation of the metabolic syndrome, is characterized by a wide spectrum of liver phenotypes ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), the aggressive form of the disease leading to liver fibrosis and finally cirrhosis with its life threatening complications. The evolution of NAFLD to cirrhosis is not mandatory: around 20-30% of NAFLD patients develop NASH with only some of them further evolving to fibrosis and then cirrhosis (1, 2). NAFLD is a complex disease driven by the interaction between host genetic background and environmental factors. Genetic polymorphisms explain a small part of the inter-individual variability in hepatic phenotypes observed in NAFLD patients. As an example, the well-known I148M variant of the PNPLA3 gene is associated only with a 3.5-fold greater risk of NASH, and a 3.2-fold higher risk of developing liver fibrosis (3). Several other factors that influence the course of the disease have been identified such as epigenetics (4), hormonal status (5), or nutrition (6). However, despite progress in knowledge about NAFLD pathogenesis, the fact that some NAFLD patients develop NASH/fibrosis, while most of them do not, remains incompletely understood.

Recently, the gut microbiota has gained great attention in metabolic diseases since gut dysbiosis has been demonstrated in obesity (7, 8), the metabolic syndrome (9, 10), diabetes (11, 12), and cardiovascular diseases (13). Recent animal studies have placed the gut microbiota as a potentially important player in the pathogenesis of NAFLD (14, 15). However, data linking gut dysbiosis with the severity of NAFLD remains poorly documented in humans. Only a few series with generally small sample sizes, heterogeneous populations (adult versus children), and different methods for gut microbiota evaluation (qPCR versus pyrosequencing) are available in the literature (16-18). In addition, because liver biopsy was not available in all patients to phenotype liver lesions, under-diagnosis of NASH was possible, especially in obese patients (18). Finally, these studies focused on NASH and hence, very few patients with liver fibrosis were included, limiting assessment of the association between gut dysbiosis and fibrosis in NAFLD.

The aim of the present study was to evaluate if the severity of NAFLD lesions, i.e. NASH and fibrosis, is associated with gut dysbiosis, in a well-characterized and well-balanced population of biopsy-proven NAFLD patients.

PATIENTS AND METHODS

Patients

Patients with biopsy-proven NAFLD were consecutively included from October 2012 to September 2013 at Angers University Hospital (France). NAFLD was defined as liver steatosis on liver biopsy after exclusion of concomitant steatosis-inducing drugs, excessive alcohol consumption (>210 g/week in men or >140 g/week in women), chronic hepatitis B or C infection, and histological evidence of other concomitant chronic liver disease. Patients were excluded if they had cirrhosis complications (ascites, variceal bleeding, systemic infection, or hepatocellular carcinoma), history of chronic inflammatory bowel disease or bariatric surgery, or if they had been treated with antibiotics within the 2 months before inclusion. The study protocol conformed to the ethical guidelines of the current Declaration of Helsinki and was approved by the local ethics committee. All patients gave informed written consent before participating to the study.

Liver histology

Pathological examination of liver biopsy was performed by an expert of the NASH-CRN network (CG) who was blinded for patient data. Steatosis, lobular inflammation, ballooning, and liver fibrosis were semi-quantitatively evaluated according to the NASH CRN scoring system (19): F0 = no fibrosis, F1 = perisinusoidal or portal/periportal fibrosis, F2 = perisinusoidal and portal/periportal fibrosis, F3 = bridging fibrosis, and F4 = cirrhosis. As recently recommended (20), NASH was defined as the presence of each of the 3 following conditions: steatosis grade ≥1, lobular inflammation grade ≥1, and ballooning grade ≥1. ‘Significant fibrosis’ was defined as fibrosis stage F≥2.

Stool sample collection, microbial DNA extraction, amplicon library construction and sequencing

Stool samples were collected the day of the liver biopsy and immediately frozen at −80°C. Genomic DNA was isolated from stool samples using the PowerSoil DNA isolation kit (Mobio Laboratories) following the manufacturer's protocol.

DNA sequencing—Amplicon libraries were constructed with Illumina sequencing-compatible and barcode-indexed bacterial/archael PCR primers 515F and 806R, which target the V4 region of 16S rRNA gene (21). All PCR reactions were performed with Kappa HiFi using the manufacturer's protocol (Kappa Biosystems) and approximately 50 ng of extracted DNA per reaction. Reactions were held at 94°C for 3 min followed by 35 cycles of 94°C × 45 sec, 50°C × 60 sec, and 72°C × 90 sec. A final 10 min 72°C extension completed the reactions. All amplicons were purified by gel extraction (E-Gel; Invitrogen). The purified amplicons were then pooled in equimolar concentrations and the final concentration of the library was determined by Qubit (Invitrogen). Amplicon libraries were mixed with 30%

PhiX control DNA. Sequencing was performed on a MiSeq instrument (Illumina) using a 250 × 2 V2 kit.

Clustering MiSeq reads into operational taxonomic units—Paired end reads were first merged and de-multiplexed into patient samples using Qiime version 1.9 (22). Subsequent processing of amplicon sequences was performed with UPARSE version 7.0 (23), and included read error correction, de-replication, chimera filtering, and finally *de novo* clustering into operational taxonomic units (OTUs) at a 97% identity cut-off. Taxonomic affiliation of each OTUs was performed with QIIME against the Greengenes database version 13.8.

Inferred metagenomics prediction of stool samples

A predicted functional composition of the gut microbiome was inferred for each stool samples using PICRUSt. Based on the fact that phylogeny and function are closely linked, this method accurately predicts the abundance of gene families from the 16S rRNA information (24). A previous study has shown that the PICRUSt imputed and shotgun sequenced metagenomes have very good correlation with an average Spearman coefficient around 0.8 (24). Briefly, metagenome inference was performed with 16S rRNA gene sequences clustered at a 97% identity threshold using closed reference of the Greengenes version 13.5 database. The resulting OTU table was then normalized by 16S rRNA gene copy number and predicted gene family abundance was inferred for each sample. Significant functional differences between patient classes were assessed with LEfSE (25) using a p value ≤ 0.05 and a LDA score >2.

Statistics

Quantitative variables were expressed as median with 1st and 3rd quartiles into brackets. Raw observation counts in taxa summary plots were normalized by calculating relative abundance. Qualitative variables were compared using the Fisher's exact test and quantitative variable using the Mann Whitney test. A p value less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 18.0 software (IBM, Armonk, NY, USA).

RESULTS

Patients

57 NAFLD patients were included in the study. Their characteristics are detailed in **Table 1**. Median age was 60 years and 34 patients (60%) were male. Forty six patients (81%) had a metabolic syndrome and 23 (40%) were diabetics under treatment. Thirty patients (53%) had F0/1 fibrosis stage on liver biopsy, of whom 10 had NASH. The remaining 27 patients (47%) had significant fibrosis (F≥2). Two patients with F≥2 fibrosis stage (respectively F3 and F4) had no NASH because no ballooning was demonstrated on liver biopsy. Fibrosis stage repartition from F0 to F4 was, respectively; 16, 14, 15, 6, and 6 patients.

Gut microbiota

After paired end read merging and error correction of 16S rRNA sequencing, a total of 10,896,711 high quality sequences were obtained from the 57 stool samples with a mean of 191,170 ± 12,585 sequences per sample (range: 21,978 – 554,352). Based on ≥97% sequence identity, amplicons were clustered into 2,371 OTUs of whom 2,269 were finally assigned using the Greengenes database and 102 unassigned. The OTU richness and phylogenic diversity from the gut microbiota were not associated with NAFLD severity (see **Figures s1 and s2** in Supplementary Material). Twelve bacteria phyla, 65 families and 133 genera were identified in the gut microbiomes in this study. At the phylum level, the taxonomic composition of the gut microbiomes showed no difference according to increasing NAFLD severity (**Figure 1a**). Significant differences started to appear at the family level (**Figure 1b**): Bacteroidaceae increased with the severity of liver lesions, whereas Prevotellaceae and Erysipelotrichaceae decreased.

Gut dysbiosis and NASH

Two genera, *Bacteroides* and *Prevotella*, significantly differed between patients with NASH and those without (**Table 2**). Compared to those without NASH, patients with NASH had higher abundance of *Bacteroides* and lower abundance of *Prevotella* (**Figures 2a and 2b**). As previously described (26), these 2 genera act as competitors with an inverse relationship between their respective abundance (**Figure 2c**). Multivariate analysis adjusted on metabolic factors (BMI, diabetes, elevated blood pressure, elevated triglycerides, reduced HDL-cholesterol, metabolic syndrome) showed that *Bacteroides* abundance was independently associated with NASH (**Table s1** in Supplementary Material). The study sample of 57 patients was divided according to the tertiles of *Bacteroides* count (**Figure 2d**): patients in the tertiles 2 and 3 had a 2-fold increase in NASH compared to those in the first tertile (74% vs 37%, p=0.010).

Gut dysbiosis and significant F≥2 fibrosis

The 3 genera *Bacteroides*, *Prevotella*, and *Ruminococcus* significantly differed between patients with F0/1 fibrosis and those with significant F≥2 fibrosis (**Table 3**). Compared to F0/1 patients, those with F≥2 fibrosis had higher abundances of *Bacteroides* and *Ruminococcus*, and lower abundance of *Prevotella* (**Figure 3a-c**). Multivariate analysis adjusted on metabolic factors showed that *Ruminococcus* abundance was independently associated with fibrosis F≥2 (**Table s2** in Supplementary Material). The study sample was divided according to the tertiles of *Ruminococcus* count (**Figure 3d**): patients in the third tertile had a 2-fold increase in fibrosis F≥2 compared to those in the tertiles 1 and 2 (74% vs 34%, p=0.010).

Bacteroides, *Ruminococcus*, and increasing severity of NAFLD lesions

As *Bacteroides* were independently associated with NASH and *Ruminococcus* with fibrosis F≥2, we evaluated the severity of NAFLD lesions according to 3 subgroups defined by the level of these 2 bacteria: *Bacteroides* <38% (tertile 1), *Bacteroides* ≥38% (tertiles 1-2) and *Ruminococcus* ≤1.4% (tertiles 1-2), *Bacteroides* ≥38% and *Ruminococcus* >1.4% (tertile 3). The rate of NASH in these 3 subgroups was, respectively: 37%, 71%, and 79% (p=0.024,

Figure 4a). The rate of fibrosis F≥2 was, respectively: 32%, 42%, and 79% (p=0.021, **Figure 4a**). Thus, using the abundance of *Bacteroides* and *Ruminococcus*, it was possible to define 3 distinct subgroups with increasing NAFLD severity: low NASH/low fibrosis, high NASH/low fibrosis, and high NASH/high fibrosis (**Figure 4a**).

As previously described (27), the presence of the metabolic syndrome was strongly associated with more severe NAFLD lesions (**Figure 4b**). We evaluated whether the levels of *Bacteroides* and *Ruminococcus* help to stratify the 2 metabolic syndrome categories (no/yes) in several subgroups with increasing severity of NAFLD. Three of the 11 patients without metabolic syndrome had fibrosis F≥2. Stratification of these 11 patients in 3 subgroups according to the level of *Bacteroides* and *Ruminococcus* did not lead to significantly different rates of fibrosis F≥2 patients (**Figure 4b**). Of the 46 patients with a metabolic syndrome, 12 had fibrosis F0/1 without NASH, 10 had fibrosis F0/1 with NASH, and 24 had fibrosis F≥2. The stratification of these 46 patients according to *Bacteroides* and *Ruminococcus* abundance led to subgroups with significantly increased severity of NAFLD lesions (**Figure 4b**). The same pattern was observed when diabetes or the various components of the metabolic syndrome were considered instead of the metabolic syndrome (**Table s3**).

Inferred metagenome content of stool samples

The functional potential of bacterial assemblages associated to each stool sample was predicted with PICRUSt using level 3 of KEGG orthologs. As assessed with LEfSE at a p value <0.05, the gut microbiome of NASH patients was significantly enriched in 6 functional categories compared to patients without NASH. These enriched functional categories (**Table 4**) were related to carbohydrate metabolism (e.g. glyoxylate/dicarboxylate metabolism, starch/sucrose metabolism), lipid metabolism (e.g. sphingolipid metabolism), amino acid metabolism (e.g. cyanoamino acid metabolism) and secondary metabolism (e.g. phenylpropanoid biosynthesis). Compared to F0/1 patients, the gut microbiome of patients with significant F≥2 fibrosis was significantly enriched in 6 functional categories. These enriched categories (**Table 4**) were also related to carbohydrate metabolism (e.g. glyoxylate/dicarboxylate metabolism, pentose and glucuronate interconversions, pentose phosphate pathway) and lipid metabolism (e.g. fatty acid biosynthesis, lipid biosynthesis proteins).

DISCUSSION

Recent evidence has linked gut microbiota to the pathogenesis of NAFLD (14, 15). Indeed, by manipulating the gut microbiota, animal studies have demonstrated direct roles for gut microbiota in each of the liver lesions observed in NAFLD: steatosis (28), NASH (29), fibrosis (30), and liver cancer (31). However, human data in this field remain scarce in the literature. Our work has several strengths compared to previously published human studies that have evaluated gut dysbiosis in NAFLD (16-18). First, our population was well phenotyped for liver lesions since each patient enrolled underwent a diagnostic liver biopsy. Because NASH is poorly diagnosed by usual clinical and serological parameters, studies that have compared individuals with biopsy-proven NASH to an un-biopsied obese control group might have been biased by un-diagnosed NASH in controls. In addition, our population

encompassed the entire spectrum of non-malignant liver lesions observed in NAFLD, i.e. steatosis, NASH, and fibrosis. This permitted us to determine if particular gut microbiota profiles associate with different liver phenotypes of NAFLD, and to evaluate for the first time in human NAFLD the association between gut dysbiosis and liver fibrosis. Finally, this is the first work to study gut dysbiosis in adult NAFLD patients from the European continent. Our results show that the more serious NAFLD lesions (i.e., NASH and significant fibrosis) associate with gut dysbiosis. More specifically, we found that an increased abundance of *Bacteroides* genus independently associated with NASH, and that increased abundance in *Ruminococcus* was independently associated with fibrosis. As discussed below, this dysbiosis shifted the metabolic potential of the gut microbiota, thereby potentially altering host exposure to various factors that have been linked to NAFLD pathogenesis

NAFLD lesions are more severe in patients with the metabolic syndrome than those without it (32). Indeed, the latest AASLD guidelines state that the presence of the metabolic syndrome can be used to identify NAFLD patients in whom liver biopsy is particularly justified (27). Despite the relatively small sample size, our study revealed an association between *Bacteroides* abundance in the gut and NASH, and this relationship was independent of metabolic factors (metabolic syndrome, BMI, diabetes, elevated blood pressure, elevated triglycerides, reduced HDL-cholesterol). Further, within the patients who had a metabolic syndrome, the severity of NAFLD lesions significantly increased as a function of the 3 subgroups defined by *Bacteroides* and *Ruminococcus* abundance. These results show that gut microbiota analysis adds prognostic information to the classical risk factors for NAFLD severity, and strongly suggests that the gut microbiota has a significant role in the pathogenesis of human NAFLD. As NAFLD is a complex disease resulting from the interaction of several factors, further studies are required to determine how members of the gut microbiota, environmental factors such as nutrition, and host genetics interact to modulate NAFLD pathogenesis. This knowledge will enable more precise profiling of NAFLD patients who are at risk for progressive liver damage.

Published literature suggests several mechanisms that may explain why increased *Bacteroides* abundance in the gut promotes NASH. *Bacteroides* abundance displays strong positive correlations with the fecal content of deoxycholic acid, D-pinitol, choline, raffinose and stachyose (the two last contain glucose and fructose). Conversely, negative correlations between fecal *Bacteroides* and fecal short chain fatty acids (SCFA) and amino acids have been reported (33). Most of these compounds influence the pathogenesis of NASH. For example, deoxycholic acid induces apoptosis in rat liver, and is increased in the livers of NASH patients (34, 35). Fructose has been associated with increased liver inflammation and fibrosis in NAFLD patient (36). Hence, *Bacteroides*-associated increases in deoxycholic acid, raffinose, and stachyose are predicted to promote NASH, while decreased SCFAs might be detrimental for NAFLD (37, 38).

We also observed that increases in fecal *Bacteroides* abundance were paralleled by decreases in *Prevotella*. This finding is consistent with evidence that *Bacteroides* and *Prevotella* are competitors. Dietary composition is known to influence the balance between *Bacteroides* and *Prevotella* in the gut: Western diets rich in fat, animal proteins and sugar favor

Bacteroides; while agrarian society diets that are rich in fiber, starch and plant polysaccharides promote *Prevotella* abundance (26, 39-41). Western-type diets high in fructose and saturated fats have been associated with NASH. Thus, evidence of increased *Bacteroides* and decreased *Prevotella* in our NASH patients is in line with previously published information about the relationship between diet and human NASH (6). A mechanism that might explain this relationship was identified by a recent study that evaluated rapid and diet-specific alterations of gut microbial communities under a 'plant-based' or an 'animal-based' diet (42). Namely, the animal-based diet rapidly induced a shift of gut microbial community to favor *Bacteroides* abundance. *Bacteroides* accumulation correlated with an accumulation of branched-chain fatty acids that are produced by amino acid fermentation. The latter are known to promote insulin-resistance (43), and insulin resistance increases the risk for NASH.

Our results also demonstrated an independent, positive correlation between *Ruminococcus* abundance and significant (F≥2) liver fibrosis. There is little published information to guide hypotheses generation about this association. *Ruminococcus* are able to ferment complex carbohydrates such as cellulose, pectine, resistant starch (44, 45), and are acetate and propionate producers (46, 47). The *Ruminococcus* genus is quite heterogeneous, including both beneficial and deleterious bacteria. For example, *Ruminococcus* bromii is known to have beneficial effects on health (48), while other *Ruminococcus* species have been shown to be pro-inflammatory (49, 50). In a recent study, *Ruminococcus* abundance increased in formula-fed infant rhesus monkeys, and this was accompanied by increased branched-chain amino acids, hyperinsulinemia, and an inflammatory state (51). *Ruminococcus* are also able to produce alcohol (46) and this might have detrimental actions on intestinal permeability and hepatic inflammation. As with *Bacteroides*, *Ruminococcus* abundance in the gut seems to be influenced by diet composition, but the effects of diet appear to be complex. For example, animal based diets increase *Ruminococcus gnavus* but decrease *Ruminococcus bromii* and *Ruminococcus callidus* (42).

To further explore hypotheses linking *Bacteroides* and *Ruminoccocus* to the severity of NAFLD, we used PICRUSt to estimate the metagenomic profile of the gut microbiota from our patients (24). Interestingly, this functional approach showed that more serious NAFLD lesions (i.e., NASH and significant fibrosis) associated with significant shifts in the metabolic function of the gut microbiota, mainly impacting KEGG pathways that relate to metabolism of carbohydrates, lipids, and amino acids. These results provide exciting, new insights about potential roles of gut microbiota in NAFLD pathogenesis. They must be confirmed by further 'classical' metagenomics studies to precisely identify which metabolic pathways of the gut microbiota associate with NASH and/or fibrosis and thus, might promote NAFLD progression.

One limitation of our study is the relative small sample size which didn't allow us to demonstrate that small variations in the bacterial counts were statistically significant. However, we may assume that the bigger the difference between the subgroups studied, the stronger the potential effect of the bacteria on the phenotype should be. Consequently, the present work provides relevant information about the potential role of gut microbiota in the processes that drive NAFLD to the severe form of the disease, i.e. NASH and fibrosis.

In conclusion, histologic subtypes of NAFLD that increase the risk for liver-related morbidity and mortality associate with gut dysbiosis and altered metabolic functions of the gut microbiota. Enrichment of the fecal microbiome with *Bacteroides* independently and positively associates with NASH, and *Ruminococcus* accumulation similarly correlates with fibrosis stage, showing that gut microbiota analysis adds information to classical predictors of NAFLD severity. Further studies will have to decipher how metabolic functions of the gut microbiota might contribute to NASH and fibrosis in NAFLD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
OTU	operational taxonomic unit
SCFA	short chain fatty acid

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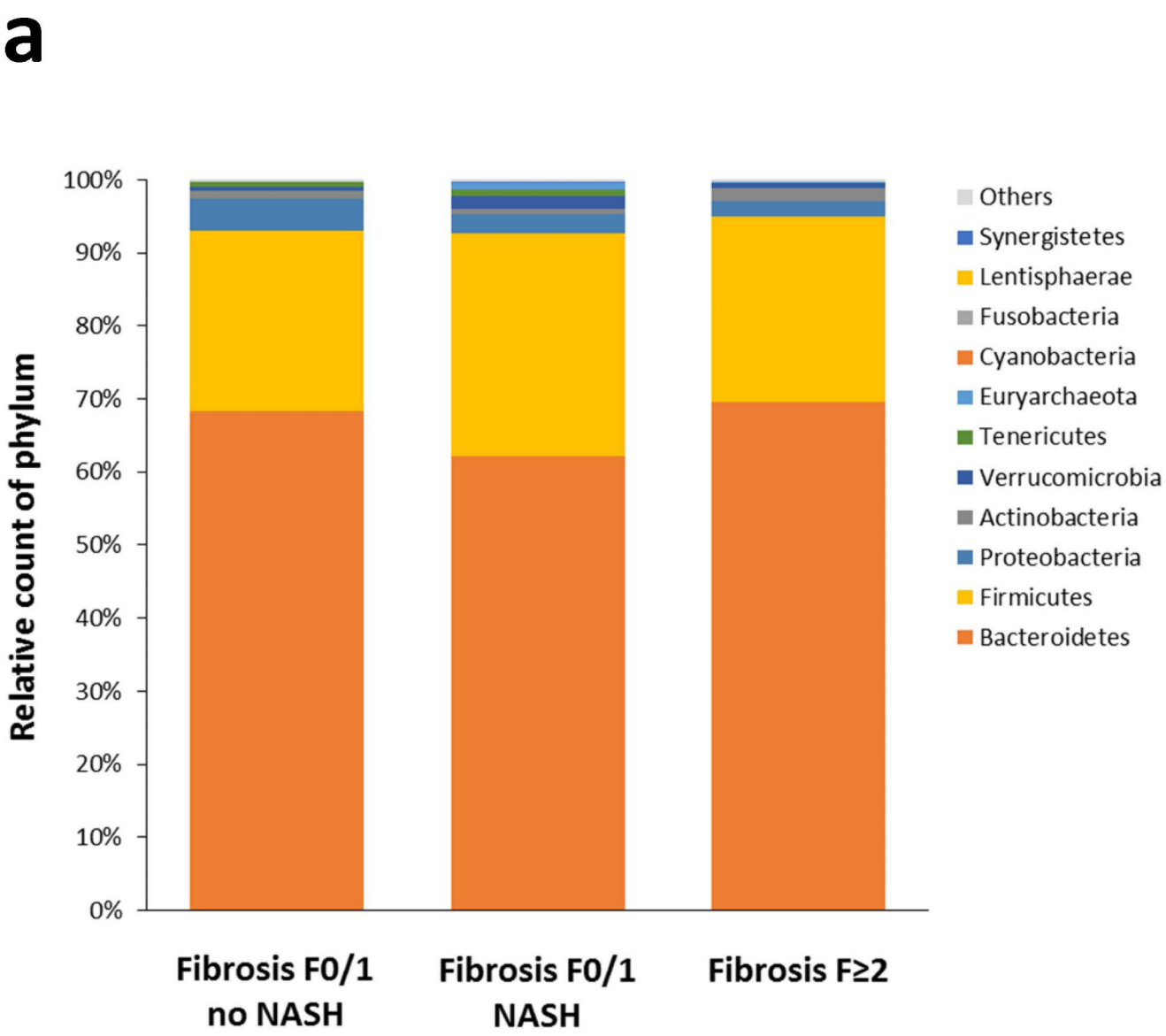

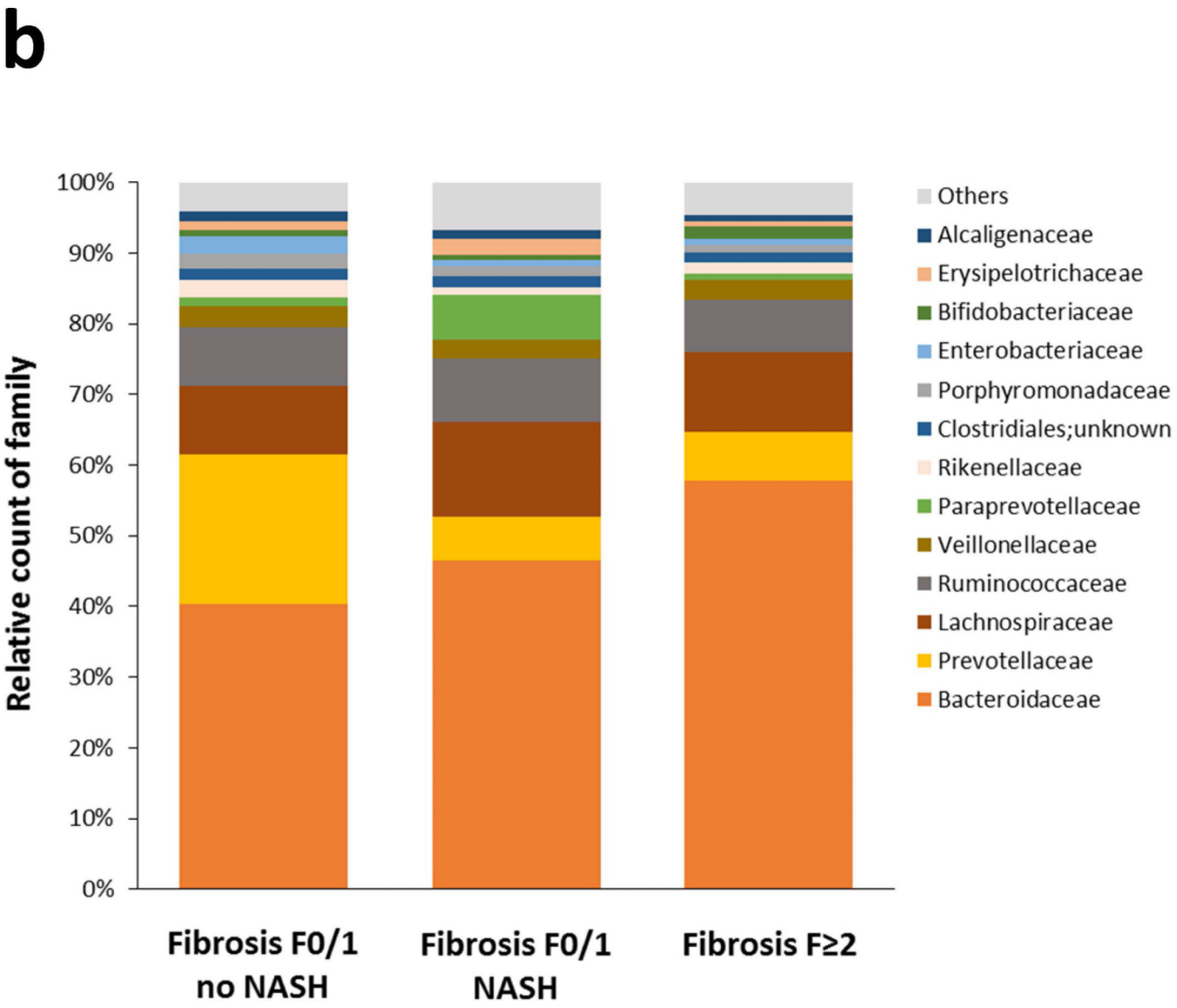

Figure 1. Taxonomic composition of the gut microbiota as a function of NAFLD severity

No significant difference was observed at the phylum level (***Figure 1a***). Significant differences appeared from the family level (***Figure 1b***).

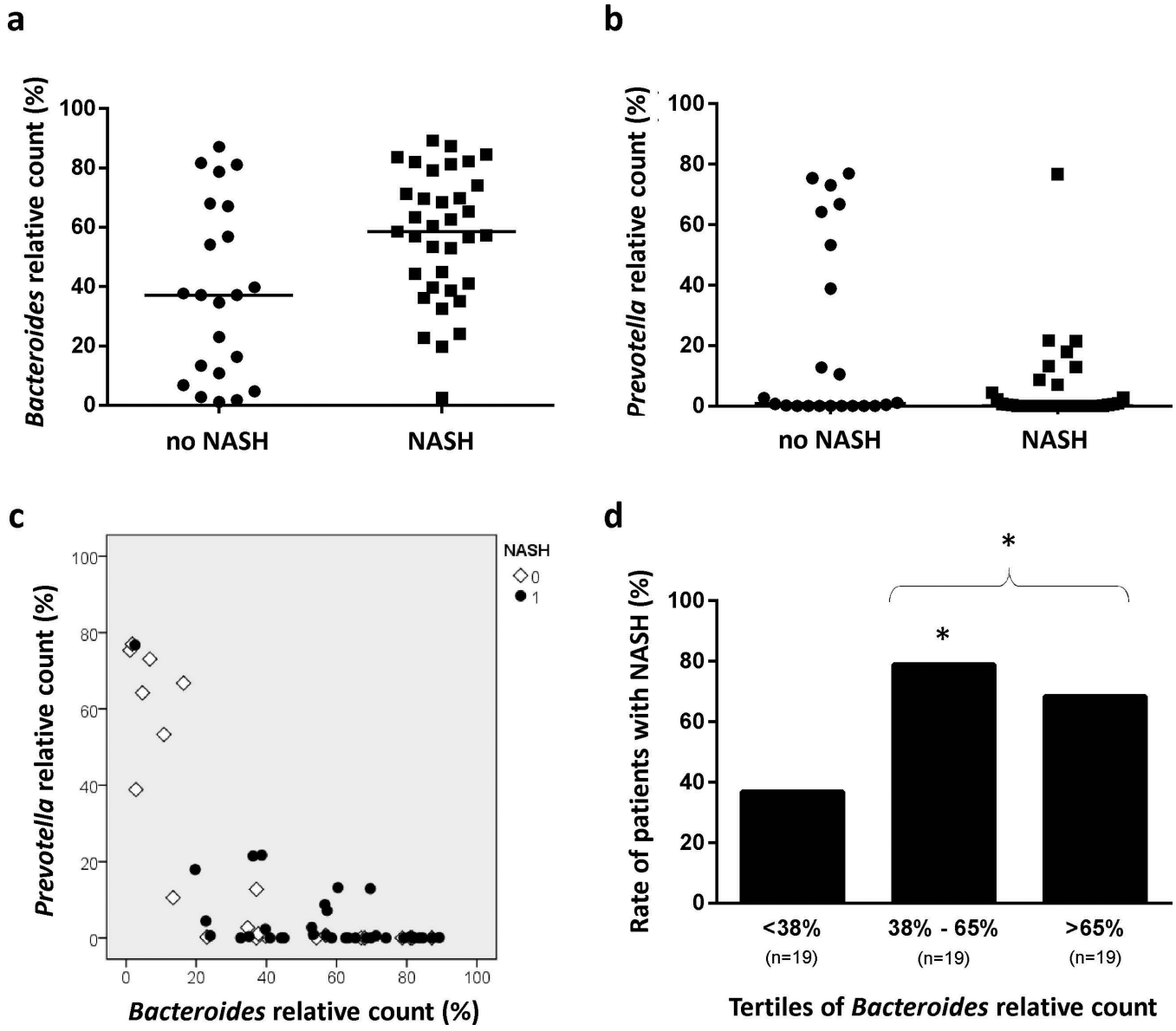

Figure 2. Relationship between NASH at liver biopsy and *Bacteroides* or *Prevotella* abundance in the gut

Figures 2a/2b: NASH patients had higher abundance of gut *Bacteroides* (p=0.013) and lower abundance of *Prevotella* (p=0.053). ***Figure 2c:*** As these bacteria act as competitors, *Bacteroides* and *Prevotella* abundance had an inverse relationship. ***Figure 2d:*** Rate of NASH patients as a function of the tertiles of *Bacteroides* relative count. The rate of NASH was significantly lower in patients with low abundance of *Bacteroides* (1st tertile). * p≤0.02 vs 1st tertile.

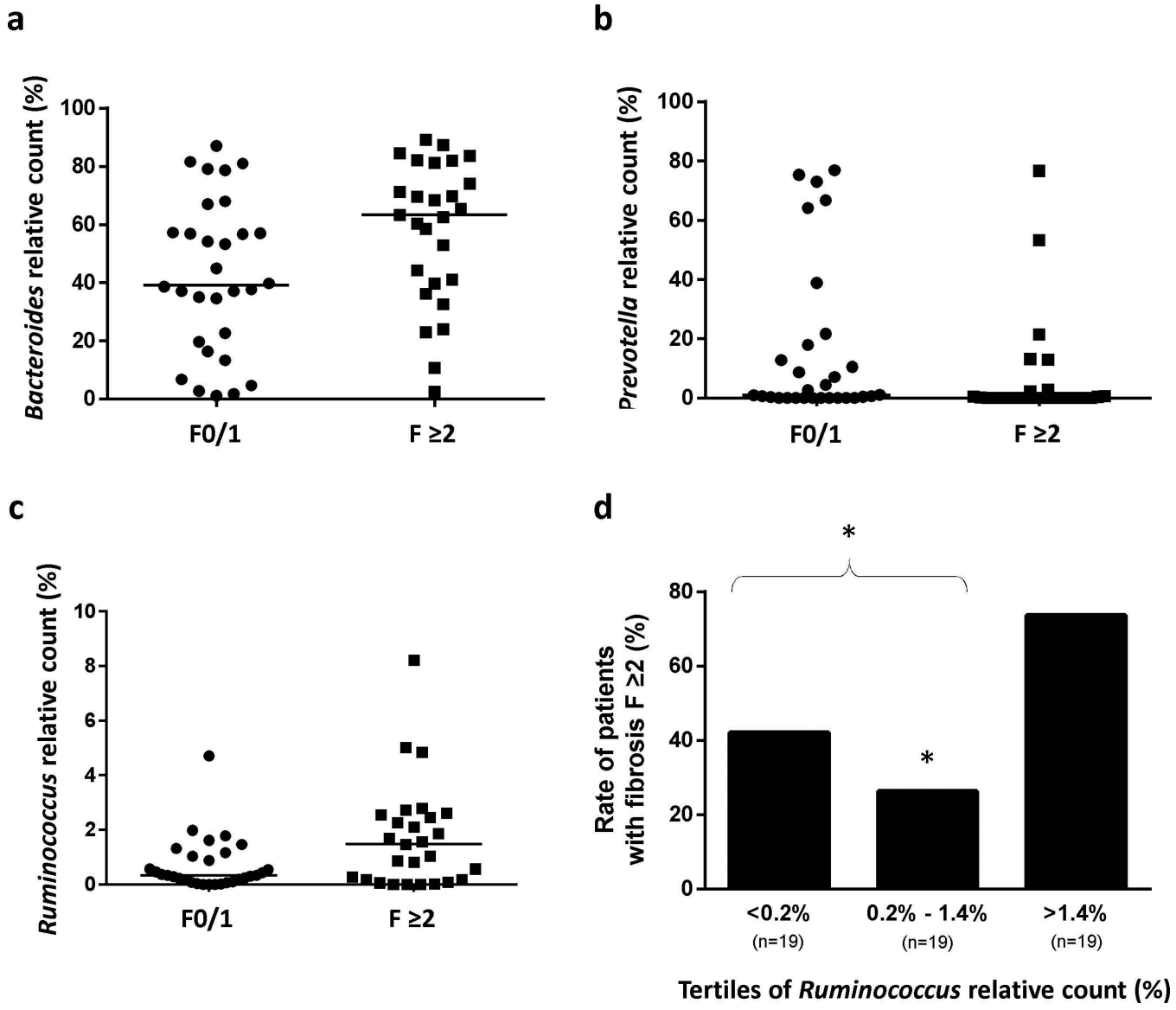

Figure 3. Relationship between significant F≥2 fibrosis at liver biopsy and *Bacteroides, Prevotella, or Ruminococcus* abundance in the gut

Figures 3a-c: Patients with fibrosis F≥2 had higher abundance of gut *Bacteroides* (p=0.018) and *Ruminococcus* (p=0.037), and lower abundance of *Prevotella* (p=0.017). ***Figure 3d:*** Rate of F≥2 patients as a function of the tertiles of *Ruminococcus* relative count. The rate of F≥2 fibrosis was significantly higher in patients with a high abundance of *Ruminococcus* (3rd tertile). * p≤0.01 vs 3rd tertile.

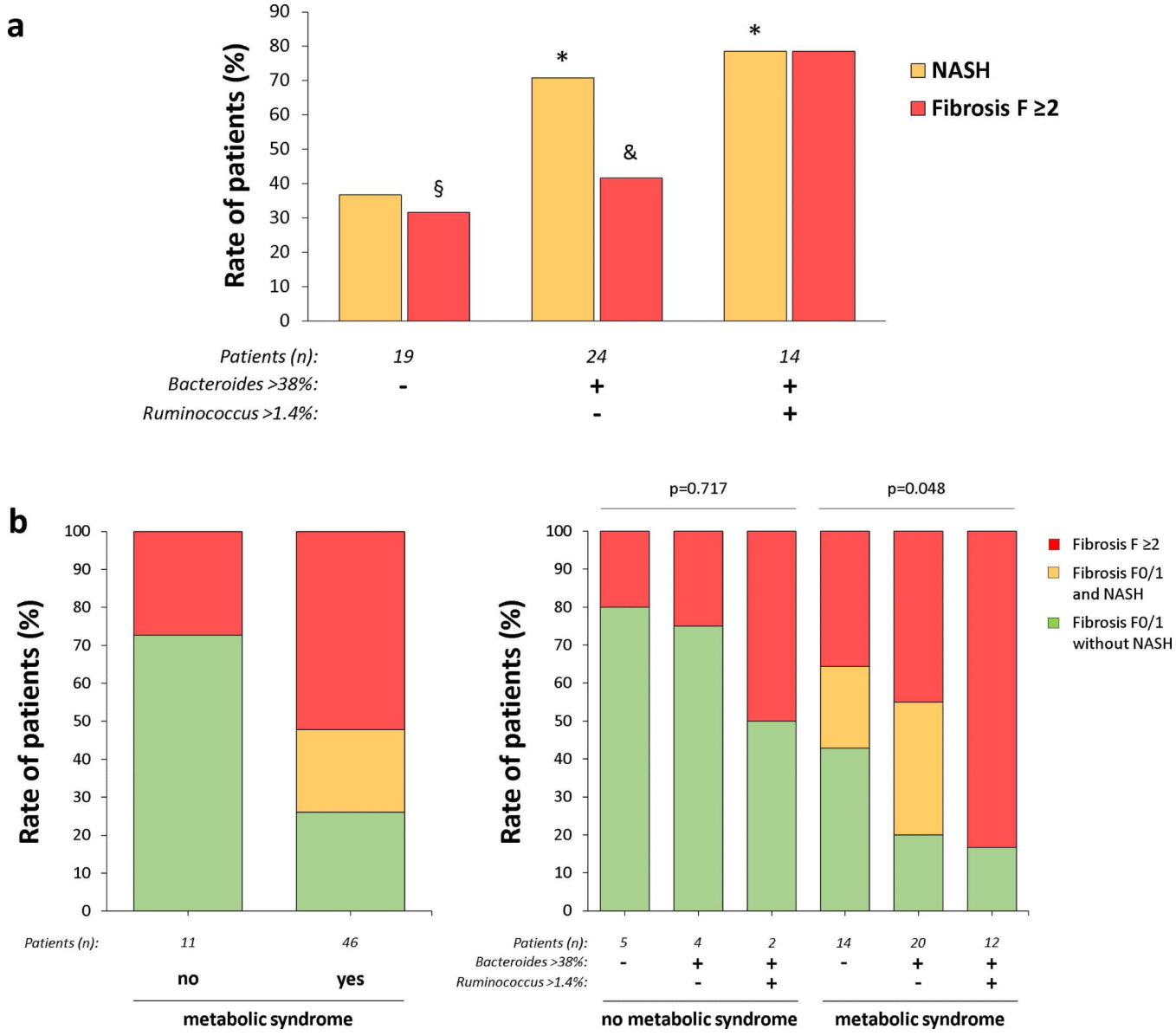

Figure 4. Severity of NAFLD lesions according to *Bacteroides* and *Ruminococcus* abundance

Figure 4a: Rate of patients with NASH or fibrosis F≥2 according to *Bacteroides* and *Ruminoccocus* abundance (* p<0.04 vs 1st subgroup, § p=0,013 vs 3rd subgroup, & p=0,043 vs 3rd subgroup). ***Figure 4b:*** Severity of NAFLD lesions according to the metabolic syndrome status alone or stratified according to the abundance of *Bacteroides* and *Ruminococcus.*

Table 1

patient characteristics at inclusion

	All	Fibrosis F0/1		Fibrosis F≥2	p
		No NASH	NASH		
Patients (n)	57	20	10	27	-
Age (years)	60 (51-66)	55 (47-64)	61 (52-70)	62 (56-67)	0.283
Sex (M/F)	34/23	13/7	6/4	15/12	0.808
BMI (kg/m^2)	31 (28-34)	30 (26-34)	31 (28-32)	32 (29-35)	0.268
Diabetes (no/yes)[a]	30/27	18/2	5/5	7/20	<0.001
Elevated blood pressure (no/yes)[b]	14/43	10/10	1/9	3/24	0.005
Elevated triglycerides (no/yes)[c]	28/29	15/5	4/6	9/18	0.015
Reduced HDL-cholesterol (no/yes)[d]	14/43	6/14	1/9	7/20	0.475
Metabolic syndrome (no/yes)	11/46	8/12	0/10	3/24	0.011
AST (IU/l)	40 (32-59)	36 (30-43)	32 (20-44)	56 (37-71)	0.002
ALT (IU/l)	64 (39-89)	62 (29-83)	44 (32-86)	69 (55-102)	0.129
GammaGT (IU/l)	80 (40-124)	71 (39-127)	53 (29-117)	93 (60-159)	0.238
Us CRP (mg/l)	1.8 (1.0-4.3)	1.4 (0.6-2.5)	2.9 (1.1-4.2)	1.8 (1.3-5.8)	0.115

Quantitative results are expressed as median with 1st and 3rd quartiles into brackets

According to the International Diabetes Federation definition:

[a]Either anti-diabetic treatment or fasting glycemia ≥7.0 mmol/l

[b]Antihypertensive treatment or elevated blood pressure with systolic ≥130 mm Hg and/or diastolic ≥85 mm Hg

[c]Lipid-lowering treatment or triglycerides ≥1.7 mmol/l

[d]Lipid-lowering treatment or HDL-cholesterol <1.1 mmol/l in men or <1.3 mmol/l in women

Table 2
Mean abundance of gut microbiome taxa in patients with or without NASH

Phyla, families, and genera with >1% occurrence in the whole population are presented.

Bacteria	No NASH (n=22)	NASH (n=35)	p[a]
Actinobacteria	**0.9**	**1.6**	**0.818**
Bifidobacteriaceae	0.9	1.6	0.511
Bifidobacterium	*0.9*	*1.6*	*0.511*
Bacteroidetes	**67.3**	**68.1**	**0.768**
Bacteroidaceae	38.3	56.9	0.013
Bacteroides	*38.3*	*56.9*	*0.013*
Porphyromonadaceae	2.0	1.2	0.577
Parabacteroides	*2.0*	*1.2*	*0.577*
Prevotellaceae	21.7	5.5	0.053
Prevotella	*21.7*	*5.5*	*0.053*
Rikenellaceae	2.2	1.5	0.987
Paraprevotellaceae	1.0	2.4	0.882
Firmicutes	**26.2**	**26.1**	**0.987**
Clostridiales; unknown[b]	2.0	1.3	0.376
Lachnospiraceae	10.7	11.3	0.491
Blautia	*1.6*	*1.9*	*0.149*
Unknown[b]	*5.4*	*5.4*	*0.451*
Ruminococcaceae	8.6	7.8	0.544
Ruminococcus	*0.8*	*1.4*	*0.235*
Unknown[b]	*7.0*	*5.7*	*0.376*
Veillonellaceae	2.8	2.9	0.123
Megasphaera	*1.5*	*1.5*	*0.650*
Erysipelotrichaceae	1.1	1.2	0.272
Proteobacteria	**4.0**	**2.4**	**0.491**
Alcaligenaceae	1.3	0.9	1.000
Sutterella	*1.3*	*0.9*	*1.000*
Enterobacteriaceae	2.2	1.0	0.325
Unknown[b]	*1.6*	*0.8*	*0.491*

[a] by Mann-Whitney test

[b] 16S rRNA sequence distinct from any known genera in this family/genus

Table 3

Mean abundance of gut microbiome taxa in patients with no/mild fibrosis (F0/1 stage) or significant F≥2 fibrosis

Phyla, families, and genera with >1% occurrence in the whole population are presented.

Bacteria	F0/1 (n=30)	F≥2 (n=27)	p[a]
Actinobacteria	**0.9**	**1.8**	**0.987**
Bifidobacteriaceae	0.9	1.8	0.949
Bifidobacterium	*0.9*	*1.8*	*0.949*
Bacteroidetes	**66.2**	**69.6**	**0.388**
Bacteroidaceae	42.4	57.8	0.018
Bacteroides	*42.4*	*57.8*	*0.018*
Porphyromonadaceae	1.9	1.0	0.231
Parabacteroides	*1.9*	*1.0*	*0.231*
Prevotellaceae	16.2	6.8	0.017
Prevotella	*16.2*	*6.8*	*0.017*
Rikenellaceae	2.0	1.6	0.949
Paraprevotellaceae	2.8	0.8	0.386
Firmicutes	**26.7**	**25.4**	**0.798**
Clostridiales; unknown[b]	1.7	1.4	0.270
Lachnospiraceae	10.9	11.3	0.774
Blautia	*1.9*	*1.6*	*0.975*
Unknown[b]	*4.9*	*5.9*	*0.397*
Ruminococcaceae	8.6	7.5	0.576
Ruminococcus	*0.7*	*1.7*	*0.037*
Unknown[b]	*7.2*	*5.1*	*0.250*
Veillonellaceae	2.9	2.8	0.620
Megasphaera	*1.2*	*1.9*	*0.891*
Erysipelotrichaceae	1.6	0.7	0.010
Proteobacteria	**3.8**	**2.1**	**0.129**
Alcaligenaceae	1.4	0.8	0.482
Sutterella	*1.4*	*0.8*	*0.482*
Enterobacteriaceae	1.9	1.0	0.099
Unknown[b]	*1.5*	*0.7*	*0.128*

[a] by Mann-Whitney test

[b] 16S rRNA sequence distinct from any known genera in this family/genus

Table 4

Functional profile of the gut microbiota from NASH or F ≥2 patients

KEGG pathways were inferred from 16s rRNA gene sequences using PICRUSt. Functional categories significantly enriched or depleted in NASH patients or patients with F ≥2 fibrosis were assessed with LEfSE (p ≤0.05, LDA >2). NASH and F ≥2 fibrosis were associated with microbiota enrichment in KEGG categories related to metabolic functions.

KO functional categories		NASH vs no NASH		F ≥2 vs F0/1	
Level 2	**Level 3**	**LDA**	**p-value**	**LDA**	**p-value**
Biosynthesis of Other Secondary Metabolites	Phenylpropanoid biosynthesis	2.34	0.03	-	-
Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism	2.28	0.03	2.28	0.02
Carbohydrate metabolism	Pentose and glucuronate interconversions	-	-	2.74	0.02
Carbohydrate metabolism	Pentose phosphate pathway	-	-	2.44	0.01
Carbohydrate metabolism	Starch and sucrose metabolism	2.68	0.01	-	-
Carbohydrate metabolism	Unclassified	2.22	0.02	2.27	0.01
Lipid Metabolism	Fatty acid biosynthesis	-	-	2.10	0.03
Lipid Metabolism	Lipid biosynthesis proteins	-	-	2.07	0.03
Lipid Metabolism	Sphingolipid metabolism	2.45	0.04	-	-
Metabolism of Other Amino Acids	Cyanoamino acid metabolism	2.27	0.03	-	-
Translation	Translation factors	-	-	−2.25	0.05
Replication and repair	DNA replication proteins	-	-	−2.55	0.04