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The Fecal Mycobiome in Non-alcoholic Fatty Liver Disease

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Abstract

Background & Aims: Studies investigating the gut-liver axis have largely focused on bacteria, whereas little is known about commensal fungi. We characterized fecal fungi in patients with non-alcoholic fatty liver disease (NAFLD) and investigated their role in a fecal microbiome-humanized mouse model of Western diet-induced steatohepatitis.

Methods: We performed fungal internal transcribed spacer 2 sequencing using fecal samples from 78 NAFLD patients, 16 controls and 73 patients with alcohol use disorder (AUD). Anti-*Candida albicans* (*C. albicans*) IgG was measured in blood samples from 17 controls and 79 NAFLD patients. Songbird, a novel multinominal regression tool, was used to investigate mycobiome changes. Germ-free mice were colonized with feces from patients with non-alcoholic steatohepatitis (NASH), fed a Western diet for 20 weeks and treated with the anti-fungal amphotericin B.

Results: The presence of non-obese NASH or F2-F4 fibrosis was associated with a distinct fecal mycobiome signature. Changes were characterized by an increased log-ratio for *Mucor* sp./*Saccharomyces cerevisiae (S. cerevisiae)* in patients with NASH and F2-F4 fibrosis. The *C. albicans/S. cerevisiae* log-ratio was significantly higher in non-obese patients with NASH when compared with non-obese patients with NAFL or controls. We observed a different fecal mycobiome composition in NAFLD patients with advanced fibrosis as compared with AUD patients and advanced fibrosis. Plasma anti-*C. albicans* IgG was increased in NAFLD patients with advanced fibrosis. Gnotobiotic mice, colonized with human NASH feces and treated with amphotericin B were protected from Western diet-induced steatohepatitis.

Conclusions: Non-obese NAFLD patients with more advanced disease have a different fecal mycobiome composition than non-obese NAFLD patients with mild disease. Antifungal treatment ameliorates diet-induced steatohepatitis in mice. Intestinal fungi could be an attractive target to attenuate NASH.

LAY SUMMARY

Non-alcoholic fatty liver disease is one of the most common chronic liver diseases and is associated with changes in the fecal bacterial microbiome. We show that patients with non-alcoholic fatty liver disease and more severe disease stages have a specific composition of fecal fungi and an increased systemic immune response to *Candida albicans*. In a fecal microbiome-humanized mouse model of Western-diet induced steatohepatitis, we show that treatment with antifungals reduces liver damage.

Keywords

Fungi; microbiota; microbiome; metagenomics; gut pathogens; NAFLD; NASH

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases with up to one third of people in Western countries being affected [1]. Whereas the risk for

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disease progression is low among patients with non-alcoholic fatty liver (NAFL), patients with non-alcoholic steatohepatitis (NASH) are at increased risk for disease progression including the development of liver fibrosis, cirrhosis, and hepatocellular cancer [2]. Overall, around 20% of patients with NAFLD will develop progressive liver disease [3] and so far, no treatment options other than lifestyle modifications are available.

NAFLD can be seen as the hepatic manifestation of the metabolic syndrome due to its close interrelationship with obesity, arterial hypertension, dyslipidemia, insulin resistance and type 2 diabetes. However, NAFLD occurs also in non-obese subjects, where risk factors are less clear [4].

In recent years, several studies have shown that alterations in the gut bacterial microbiota composition contribute to progression of NAFLD. Most common observations include lower relative abundance of potentially beneficial bacteria in parallel to relative overgrowth of potentially detrimental bacteria, and a lower bacterial diversity in patients with more severe NAFLD [5]. This is particularly present in non-obese subjects with NAFLD, where gut bacterial alterations were significantly associated with fibrosis [6]. Several mechanisms may explain how alterations in the gut microbiota composition modulate NAFLD. Translocation of microbes or microbial metabolites into the portal bloodstream to the liver, which is facilitated by gut barrier dysfunction, can induce a hepatic inflammatory response in patients with NAFLD. In addition, gut microbial processing of dietary and host-derived substances leads to the production of microbial-derived metabolites that can affect the host metabolism [5].

Microbiome research has predominantly focused on gut bacteria. However, the gut microbiota also contains commensal fungi, which are collectively referred to as the mycobiome. Changes in the fecal fungal microbiome have been described in alcohol-associated liver disease [7] and primary sclerosing cholangitis [8].

The aim of this study was to describe the fecal mycobiome in a well characterized NAFLD cohort, to explore associations between features of the fecal mycobiome with different histological disease stages and to investigate the pathogenic role of intestinal fungi in a fecal microbiome-humanized mouse model of Western diet-induced steatohepatitis.

MATERIAL AND METHODS

Patients

Patients with NAFLD were prospectively enrolled between March 2015 and December 2018 in the outpatient liver department of the Clinic for Gastroenterology and Hepatology, University Hospital of Cologne, Germany, as previously described [9]. In this study, a total of 78 patients with NAFLD (69 biopsy-proven patients with NAFLD and 9 patients with NAFLD cirrhosis based on characteristic clinical findings, (see criteria in the Supplementary Methods) were included. The protocol was approved by the local Ethics Commission (reference # 15-056) and written informed consent was obtained from each patient. The study was performed in accordance with the Declaration of Helsinki.

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Additional Methods are described in Supplementary Methods and Supplementary CTAT table.

RESULTS

A total number of 78 patients with NAFLD were included in the analysis of fecal samples of whom 24 had NAFL, 54 had NASH, 40 had minimal fibrosis (F0-F1) and 38 had at least moderate fibrosis (F2-F4). In addition, 16 non-obese subjects without any known disease (controls) were included. As expected, patients with NAFLD, particularly patients with NASH and/or F2-F4 fibrosis had a significantly higher body mass index (BMI), displayed features of the metabolic syndrome more commonly and had significantly altered laboratory parameters when compared with controls or patients with NAFLD and mild liver disease stage (Table 1 and Supplementary Table 1). Liver histology data of the cohort are shown in Supplementary Table 2.

To determine if fecal fungi are associated with disease severity or other clinical metadata, we performed targeted amplicon sequencing of fungi from fecal samples using primers specific to the internal transcribed spacer 2 region of the ribosomal RNA operon. We obtained 10,344 (range 1,104-30,922) clean reads per sample on average with no differences in the average reads between groups (Supplementary Fig. 1).

Liver disease severity is associated with a distinct fecal mycobiome signature in non-obese patients with NAFLD

We identified a total of 120 fungal taxa in all fecal samples. To determine the fecal fungal ß-diversity (an estimate of similarity or dissimilarity between samples), we performed principal coordinate analyses. Patients were grouped based on liver disease severity (NAFL vs. NASH and F0-F1 vs. F2-F4 fibrosis). Since previous studies have shown that associations between the gut bacterial microbiota composition and more severe liver disease are particularly observed in non-obese subjects [6], we further divided all subjects into two groups: obese (BMI 30 kg/m^2) and non-obese (BMI <30 kg/m^2).

When we compared the overall fungal microbiota composition between controls and NAFLD patients stratified based on disease severity, we did not observe significant differences in the ß-diversity of the fungal microbiota among the groups (Fig. 1A and 1B, upper panels). However, when grouping NAFLD patients based on body weight status (non-obese vs. obese), we observed that the distances between non-obese patients with NASH or F2-F4 fibrosis and patients with NAFL or F0-F1 fibrosis were significantly larger than the distance within the respective patient group, indicating a significantly different fecal mycobiome composition (P=0.006 for NASH and P=0.037 for F2-F4 fibrosis, respectively, Fig. 1A and 1B, lower panels). This association remained significant (P=0.011 for NASH and P=0.041 for F2-F4 fibrosis, respectively) even after adjusting for age, gender, daily alcohol consumption, BMI, proton pump inhibitor (PPI) use and type 2 diabetes, as potentially confounding factors. We next assessed the number of observed fungi in each specimen (species richness) and fungal alpha-diversity (estimate of similarity or dissimilarity within a sample) based on the Shannon diversity index. Although there was a

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trend towards a lower fungal richness in non-obese patients with NAFLD and more severe liver disease, we did not observe significant differences among groups (Fig. 1C and 1D).

Compositional changes of the fecal mycobiome in patients with NAFLD

To investigate specific compositional changes among groups, we performed differential multinomial regression analyses using Songbird, which was developed to overcome the limitation of comparing relative abundance across samples [10]. This analysis was adjusted for age, gender, BMI, type 2 diabetes, PPI use and alcohol consumption as potentially confounding factors and included all of the detected 120 fungal taxa. Specific taxa such as *Candida albicans* (*C. albicans*), *Mucor* sp., *Cyberlindnera jadinii*, *Penicillium* sp., unknown Pleosporales, *Babjeviella inositovora* and *Candida argentea* were associated with the presence of NASH whereas unknown Saccharomycetales and *Malassezia* sp. were associated with NAFL (Fig. 2A).

We next determined log-ratios of specific fungal taxa. Sequence reads belonging to one *S. cerevisiae* OTU were present in all samples and was therefore used as denominator. Among OTUs that could be assigned to a known fungal taxon, log-ratios for *Babjeviella inositovora/S. cerevisiae*, log-ratio *Mucor* sp.*/S. cerevisiae* as well as the log ratio for unknown *Hanseniaspora/S. cerevisiae* were significantly higher in patients with NASH compared with controls or patients with NAFL (Fig. 2B). After separating groups based on obesity, significant differences were confirmed only for non-obese subjects (Fig. 2C). The log-ratios for *C. albicans/S. cerevisiae*, unknown Pleosporales/*S. cerevisiae* and *Pichia barkeri*/*S. cerevisiae* were significantly higher in non-obese patients with NASH as compared with non-obese patients with NAFL (Fig. 2C), which was not significantly different when combining non-obese and obese patients (Fig. 2B).

For NAFLD subjects with more severe fibrosis (i.e., stages F2-F4), we observed that *Mucor* sp., *Cyberlindnera jadinii*, *C. albicans*, *Saturnispora* sp. and *Babjeviella inositovora* were positively associated with F2-F4 fibrosis in the multinomial regression analysis adjusted for clinical co-factors. OTUs belonging to *Candida argentea*, the orders Pleosparales and Saccharomycetales, and *Penicillium* sp. were more associated with F0-F1 fibrosis (Fig. 3A). Similar to what we observed when comparing NAFL and NASH, compositional changes were associated with F2-F4 fibrosis only in non-obese subjects (Fig. 3B, lower panel).

Association between diabetes and fungal taxa

Although the ß-diversity and alpha-diversity were not different between patients with NAFLD with and without type 2 diabetes (data not shown), we investigated if compositional changes are present (Supplementary Fig. 3A). The log-ratio for *Malassezia* sp./*S. cerevisiae* was significantly increased in type 2 diabetics compared with non-diabetics. In addition, the log-ratio of *C. albicans/S. cerevisiae* was significantly higher in non-obese diabetics compared with non-obese subjects with NAFLD but without type 2 diabetes (Supplementary Fig. 3B).

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Correlation between clinical variables and fungal taxa

After investigating compositional changes in the fungal microbiota among groups, we further examined correlations between log-ratios that were significant in our initial analyses (Fig. 2 and 3, Supplementary Fig. 3) and other clinical parameters. The log ratio for *C. albicans/S. cerevisiae* was significantly associated with higher alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) levels as well as lower high-density lipoprotein levels (Supplementary Fig. 4A and 4B). The log-ratio for *Mucor* sp./*S. cerevisiae* was positively associated with serum glucose levels as well as AST levels (Supplementary Fig. 4A and 4B).

Associations between compositional fecal mycobiota changes and liver histology features

We further analyzed associations between those log-ratios of fecal fungal abundance and the histological features of liver inflammation, ballooning, steatosis, NAFLD activity score (NAS) and fibrosis stage, using univariate and multivariate ordinal regression analyses. Predicted probabilities for significant associations are shown in Fig. 4. In the univariate analysis, higher log-ratios for *Mucor* sp./*S. cerevisiae* were significantly associated with the stage of fibrosis (P=0.004), the grade of liver inflammation (P=0.025) and the NAS (P=0.010), which remained significant after adjustment for age, gender, BMI, type 2 diabetes, PPI use and alcohol consumption (Fig. 4A-C). The *Cyberlindnera jadinii/S. cerevisiae log-ratio* was significantly (P=0.038) associated with the fibrosis stage in the univariate analysis; however, this did not remain significant after adjustment for co-factors (Fig. 4A). The log-ratio for *C. albicans/S. cerevisiae* was significantly associated with the hepatic inflammatory grade (P=0.029), which was more significant (P<0.001) when introducing the BMI as a moderator in the multivariate model (Fig. 4B). The log-ratio for *C. albicans*/*S. cerevisiae* was only associated with the NAS when moderating the ordinal regression model for BMI (P=0.023) (Fig. 4C). We did not observe significant associations with the grades of steatosis or ballooning (data not shown). Taken together, our data indicate that alterations in the fecal fungal microbiota are more associated with inflammatory features than with steatosis or ballooning in the liver.

Fecal mycobiome composition differs in patients with advanced fibrosis due to different liver disease etiology

To determine whether changes of the fecal mycobiome in patients with advanced fibrotic liver disease differ with respect to the liver disease etiology, fecal samples from a cohort of 73 patients with alcohol use disorder were included in the analysis. Clinical characteristics for these patients are shown in Supplementary Table 3. In the principal coordinate analysis, the distances between patients with NAFLD and F3-F4 fibrosis and patients with alcohol use disorder and F3-F4 fibrosis were significantly larger than the distance within the respective patient group, indicating a significantly (P=0.006) different fecal mycobiome composition (Supplementary Fig. 5A); the same was true when comparing non-obese subjects only (Supplementary Fig. 5B). In the Songbird analysis, adjusted for age, gender and BMI, NAFLD patients with advanced liver disease were mainly characterized by increased *Mucor* sp. whereas patients with advanced alcohol-associated liver fibrosis where mainly characterized by increased *Blumeria, Candida* and *Debaryomyces* sp. (Supplementary Fig.

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5C). These data indicates that changes in the fecal mycobiome are rather specific for different liver disease etiologies in patients with advanced liver fibrosis.

Fungi bacteria associations

We further investigated associations between fecal fungi and bacteria. In a network analysis, including bacteria and fungi that were detected in at least 10 patients, several associations were detected between different bacteria in fecal samples of patients with NAFLD, but also some associations between bacteria and specific fungi (Supplementary Fig. 6A). Among others, *C. albicans* was associated with *Eubacterium hallii* and *Bifidobacterium adolescentis*. *Cyberlindnera jadinii* was associated with *Flavonifractor plautii*, unknown Clostridiales and *Eubacterium biforme* (Supplementary Fig. 6A).

We further performed PCoA analyses for dimensionality reduction of fecal bacteria and fungi. The first three principal coordinates of bacteria and fungi as well as bacterial and fungal diversity and richness were included in random forest models in order to compare the importance of these variables for the presence of NASH and F2-F4 fibrosis. Both, bacterial and fungal features had a high importance to discriminate mild versus more advanced liver disease based on the mean decrease in gini (Supplementary Fig. 6B-C). Fungal features had an overall higher importance in non-obese patients as compared with random forest analyses including obese and non-obese patients (Supplementary Fig. 6B-C, lower panels).

Plasma antibodies specific to *C. albicans* are increased in patients with NAFLD and advanced fibrosis

Antibodies specific to *C. albicans* (Anti-*C. albicans* IgG) were measured in plasma samples from 96 subjects (17 controls and 79 patients with NAFLD) of whom 74 were overlapping with the subjects for the fecal mycobiome analysis and half-maximal effective concentrations were quantified. Clinical characteristics of these patients are shown in Supplementary Table 4. High anti-*C. albicans* IgG were significantly (P=0.022) associated with a higher fecal *C. albicans*/ *S. cerevisiae* log ratio (Fig. 5A). No significant differences of anti-*C. albicans* IgG levels were found when comparing controls, NAFL and NASH patients or controls, NAFLD F0-F1 and F2-F4 patients (data not shown). However, NAFLD patients with advanced liver fibrosis (F3-F4) had significantly higher anti-*C. albicans* IgG levels than controls or NAFLD patients with F0-F2 fibrosis (NAFLD F0-F2 vs. NAFLD F3-F4: P=0.007, controls vs. NAFLD F3-F4: P=0.007, controls vs. NAFLD F0-F2, P=0.474, Fig. 5B). Plasma anti-*C. albicans* IgG remained significantly associated with advanced fibrosis even after adjusting for total plasma IgG levels in a logistic regression analysis (Odds ratio log (anti-*C. albicans* IgG) per log unit increase: 3.17, 95%, confidence interval 1.13-8.88, P=0.029). These data indicates that NAFLD patients with advanced liver fibrosis have an increased immune response to *C. albicans*, which might be caused by increased intestinal *C. albicans* numbers and/or more frequent systemic exposure to *C. albicans*.

Antifungal treatment reduces Western diet-induced steatohepatitis and liver fibrosis in fecal microbiome-humanized gnotobiotic mice

To determine the importance of intestinal fungi in the pathogenesis of diet-induced steatohepatitis, germ-free C57BL/6 mice were transplanted ("humanized") fecal microbiota

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from patients with NASH and subsequently fed a Western diet for 20 weeks. The presence of the two fungi *C. albicans* and species of *Mucor, Rhizomucor,* and *Rhizopus (Mucor/Rhizopus spp.)*, which had the most obvious association with NASH, was confirmed in the donor stool by performing PCR analysis. Western diet-fed mice treated with the poorly absorbable antifungal amphotericin B showed a lower liver weight-to-body weight ratio (Supplemental Fig. 7A) despite increased food intake compared with the Western diet-fed mice not treated with the antifungal (Supplemental Fig. 7B). Amphotericin B-treated mice were protected from liver injury as indicated by lower ALT levels and exhibited decreased hepatic triglyceride and cholesterol concentrations as compared with Western diet-fed control mice (Fig. 6A-D). Amphotericin B also reduced hepatic inflammation, as demonstrated by lower hepatic mRNA expression of tumor necrosis factor-alpha (*Tnf-α),* chemokine C-C motif ligand-2 (*Ccl2*), and adhesion G protein-coupled receptor E1 (*Adgre1*), also known as *F4/80*, in comparison with Western diet-fed control mice without treatment (Fig. 6E-F, Supplemental Fig. 7C). Further, amphotericin B attenuated liver fibrosis, as determined by decreased hepatic mRNA expression of genes involved in fibrosis, including transforming growth factor-beta 1 (*Tgfb1*), tissue inhibitor of metalloproteinase-1 (*Timp1*), and collagen type I alpha 1 (*Col1a1*) (Fig. 6G-H, Supplementary Fig. 7D) as well as lower total hepatic hydroxyproline content as compared with untreated Western diet-fed mice not receiving amphotericin B (Fig 6I-J). Antifungal treatment reduced the fecal proportions of the species *C. albicans* and the species of *Mucor*, *Rhizomucor*, and *Rhizopus* (*Mucor/Rhizopus* spp.) as compared with Western diet-fed controls using quantitative PCR (Fig. 6K-L).

To investigate, if antifungal treatment induces changes in the intestinal bacterial microbiota, we measured total fecal bacteria and the major bacteria phyla Bacteroidetes, Firmicutes, and Proteobacteria in Western-diet fed mice by performing quantitative PCR. We did not find significant differences in the relative abundance of these bacteria among Western-diet fed mice treated with amphotericin B or control mice (Supplementary Fig. 8A-D). This is in line with our previous study using 16S rRNA sequencing in mice fed with ethanol or isocaloric diet and treated with amphotericin B [11].

Altogether, these results indicate that antifungal treatment ameliorates diet-induced steatohepatitis and liver fibrosis in fecal microbiome-humanized mice.

DISCUSSION

In this study, we investigated the fecal mycobiome in a cohort of patients with NAFLD and controls. Further, we measured plasma anti-*C. albicans* IgG of NAFLD patients and controls and investigated the role of fecal fungi in the pathogenesis of diet-induced steatohepatitis using fecal microbiome-humanized Western-diet fed mice. We found that particularly non-obese patients with NAFLD and advanced disease stages on liver biopsy had a different fecal mycobiome composition as compared with non-obese patients with NAFLD having mild liver disease. Among known fungal taxa, the *Mucor* sp./*S. cerevisiae* log-ratio as well as the *C. albicans*/ *S. cerevisiae* log ratio were independently associated with higher inflammatory activity. The log-ratio of *C. albicans/S. cerevisiae* was higher in non-obese diabetics compared with non-obese subjects with NAFLD but without type 2 diabetes.

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We observed a different fecal mycobiome composition in NAFLD patients with advanced fibrosis as compared with alcohol use disorder patients and advanced fibrosis. NAFLD patients with advanced fibrosis had significantly higher plasma levels of anti-*C. albicans* IgG. Western diet-fed gnotobiotic mice that were colonized with feces from patients with NASH and treated with amphotericin B showed reduced steatohepatitis and fibrosis.

Even though bacteria vastly outnumber fungi in the intestinal microbiota, fungal cells are typically 100-fold larger, which increases their proportional biomass [12]. The human intestine can become the source for systemic fungal pathogen-associated molecular patterns or fungal infections when the gut barrier is disrupted [13]. The prevalence of systemic antibodies against *Candida* and *S. cerevisiae* ranges from 24%-59% in cirrhotic patients [14], who are at an increased risk for fungal infections with a very high mortality [15, 16]. Spontaneous fungal peritonitis is mainly caused by *C. albicans* in cirrhotic patients [17]. These findings suggest that patients with chronic liver disease are frequently exposed to either fungal products and often develop fungal infections.

Studies investigating the mycobiome in mouse models are sparse. Results from two studies demonstrate that feeding mice with high-fat diet leads to a reduced fungal diversity [18] and compositional changes such as reduced abundance of *Alternaria* [18, 19], *Saccharomyces cerevisiae* [19], while *Candida* and *Hanseniaspora* [19] are higher in high-fat diet-fed mice as compared with chow-fed mice.

Besides increased *C. albicans* abundance in more severely diseased non-obese NAFLD patients in our study, *Mucor* sp. were positively associated with NASH, fibrosis and particularly hepatic inflammatory changes. *Mucor* sp. are classified as opportunistic pathogens which can cause mucormycosis, a systemic infection which occurs predominantly in patients with hematological malignancies undergoing chemotherapy, but also in patients with uncontrolled diabetes [20]. Interestingly, *Mucor* sp. in the fecal fungal microbiome of NAFLD patients were associated with higher blood glucose levels in our study. Beyond the role in causing mucormycosis, the role of *Mucor* sp. in the commensal mycobiota is not clear. *In vitro*, *Mucor* administration increased intestinal permeability in epithelial cell monolayers [21]. Overall, studies are needed to investigate which factors lead to increased abundance of *Mucor* sp. in NAFLD patients with more severe disease.

In line with the study by Lee et al., assessing the gut bacterial microbiome in patients with NAFLD [6], we found that several compositional alterations in the fungal microbiota were associated with NASH and F2-F4 fibrosis particularly in non-obese subjects. It is not well-known which factors are primarily responsible for disease progression in non-obese patients with NAFLD. One could speculate, that our results and the study by Lee et al. indicate that an altered fecal bacterial and fungal microbiome might represent a more important risk factor for disease progression in non-obese subjects, whereas in obese subjects, the highly increased BMI with its frequently associated metabolic diseases potentially represent the major driving force behind liver disease progression; however, more studies are needed to elucidate this association.

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Our study has several limitations. In contrast to databases for bacterial 16S rDNA gene sequencing, reference databases for fungal taxon alignment are less comprehensive and less accurate. It will be important to improve such databases in the future, especially focused on fungi known to be host-associated. The sample size of our NAFLD cohort is limited. In addition, longitudinal studies in humans are needed to investigate dynamics of intestinal fungi and their interaction with bacteria associated with NAFLD severity over time. Although the fecal mycobiome composition in NAFLD patients with advanced liver fibrosis was different as compared with alcohol use disorder patients and advanced liver fibrosis, liver biopsy was not performed in patients with alcohol use disorder and the presence of advanced fibrosis was noninvasively assessed. Since active alcohol consumption can affect the reliability of noninvasive fibrosis tests, the fibrosis stage could be overestimated.

Amphotericin B has a high selectivity for fungal cells through binding to the fungal sterol ergosterol at the fungal membrane with consecutive pore formation [22]. The sensitivity for targeting ergosterol is high, however, amphotericin B also targets the mammalian sterol cholesterol, although with a lower affinity [22]. Through this way, and due to known immunomodulatory effects involving binding to Toll-like receptors [22], it is possible, that some beneficial effects of amphotericin B are mediated by direct effects on mammalian cells and not exclusively through the antifungal activity.

In conclusion, we show that the histological disease severity in patients with NAFLD was associated with changes in the fecal mycobiome, particularly in non-obese subjects. Since anti-fungal treatment ameliorates diet-induced steatohepatitis in mice, targeting the gut mycobiome might be an attractive therapeutic target to improve disease outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement

Raw sequences from ITS2 gene sequencing were registered at NCBI under BioProject PRJNA698272 (NAFLD cohort). Raw sequences from ITS2 gene sequencing were registered at NCBI under BioProject PRJNA703732 (Alcohol use disorder cohort).

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Abbreviations:

ALT	alanine aminotransferase
AST	aspartate aminotransferase
BMI	body mass index
HbA1c	glycated hemoglobin
Ig	immunoglobulin
NAFL	non-alcoholic fatty liver
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NAS	NAFLD activity score
PPI	proton pump inhibitor.

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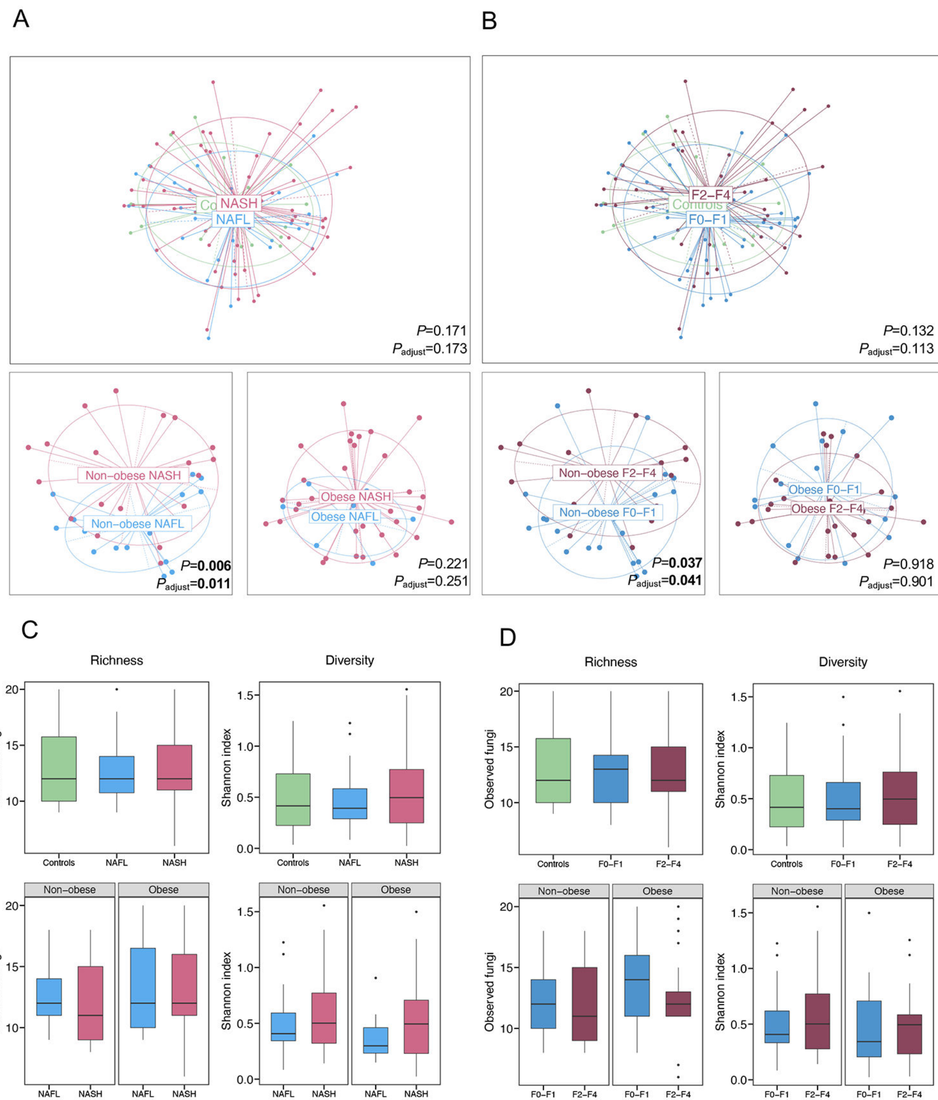

Figure 1. Liver disease severity is associated with a specific fecal mycobiome composition in non-obese patients with NAFLD.

(A-B) Principal coordinate analyses (PCoA) based on the Jaccard dissimilarity matrices for NASH and F2-F4 fibrosis. All of the detected 120 fungal taxa at were included. *P* values were determined by permutational multivariate analysis of variance (PERMANOVA) and adjusted for age, gender, BMI, type 2 diabetes, proton pump inhibitor use and alcohol consumption. Bold font indicates statistical significance ($P<0.05$). (C-D) No significant differences in the alpha diversity and richness among groups. In (A-D) 78 patients with NAFLD were included in the analysis of whom 24 had NAFL, 54 had NASH; 40 had minimal fibrosis (F0-F1) and 38 had at least moderate fibrosis (F2-F4). In addition, 16 subjects without any known disease were included. For the box and whisker plots (C-D), the box extends from the 25th to 75th percentile, with the center line indicating the median; the bottom whiskers indicate the minimum values and the top whiskers indicate the 75th percentile plus 1.5-fold the inter-quartile distance (the distance between the 25th and 75th percentiles). Values greater than this are plotted as individual dots. *P* values determined by

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Kruskal-Wallis test with Dunn's post-hoc test for skewed distributions followed by false discovery rate (FDR) or Whitney-Wilcoxon rank-sum test.

NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

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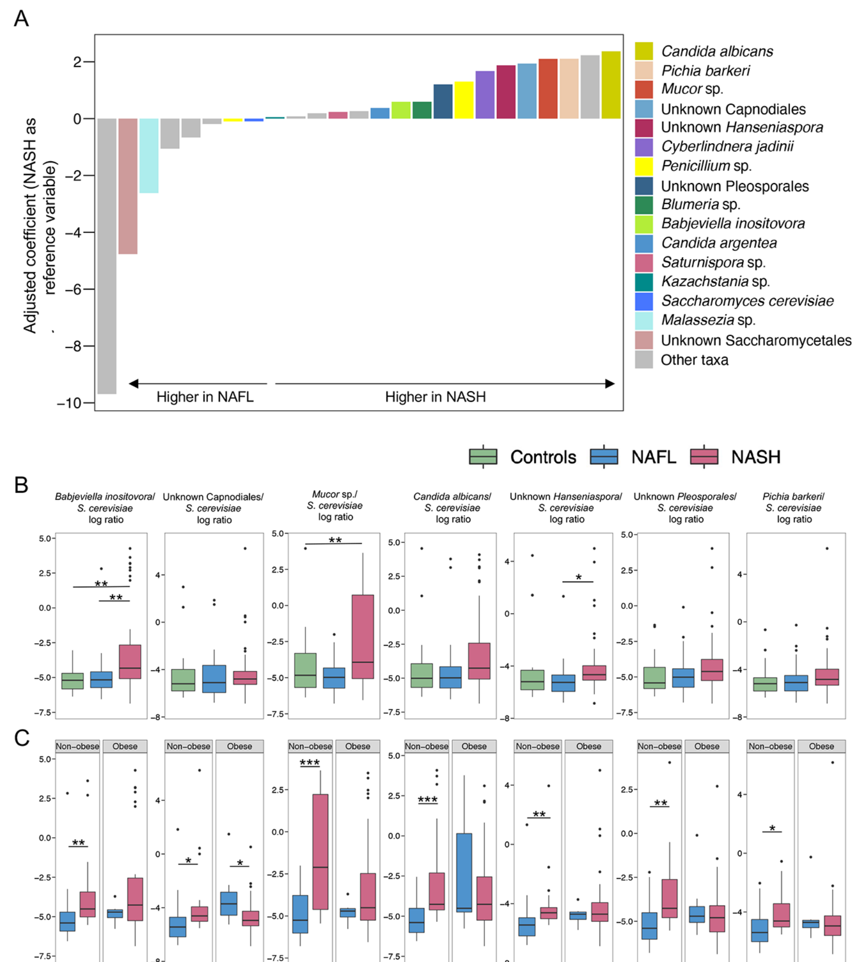

Figure 2. Compositional changes in the fecal mycobiome of patients with NASH.

(A) Differential abundance of fungal taxa among groups were calculated using Songbird under consideration of clinical metadata (age, gender, BMI, type 2 diabetes, proton pump inhibitor use and alcohol consumption). Fungal taxa with at least 90% confidence level alignment in the UNITE database were colored. (B) Log-ratios of specific fungal taxa that were significantly different among groups, either when including all patients, or (B) when investigating subgroups based on obesity. We determined the denominator for the calculation of the log-ratios by choosing a fungal taxon that is present in all samples. This was the case for *Saccharomyces cerevisiae (S. cerevisiae)*. In order to handle problems occurring when calculating log-ratios in zero-inflated microbiome data, we added a constant small value (0.1) to each count before calculating log-ratios. In (A-C), 78 patients with NAFLD were included in the analysis of whom 24 had NAFL, 54 had NASH. Of the patients with NAFL, 17 (70.8%) were non-obese and 7 (29.2%) were obese. Of the patients with NASH, 21 (38.8%) were non-obese and 33 (61.2%) were obese. In (B-C), 16 subjects without any known disease were additionally included. *P* values determined by Kruskal-Wallis test with Dunn's post-hoc test for skewed distributions followed by false discovery rate (FDR) or Whitney-Wilcoxon rank-sum test. **P*<0.05, >0.01; ***P*<0.01, *P*>0.001; ****P*<0.001. For the box and whisker plots (b-c), the box extends from the 25th to 75th percentile, with the center line indicating the median; the bottom whiskers indicate the minimum values

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and the top whiskers indicate the 75th percentile plus 1.5-fold the inter-quartile distance (the distance between the 25th and 75th percentiles). Values greater than this are plotted as individual dots.

NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis.

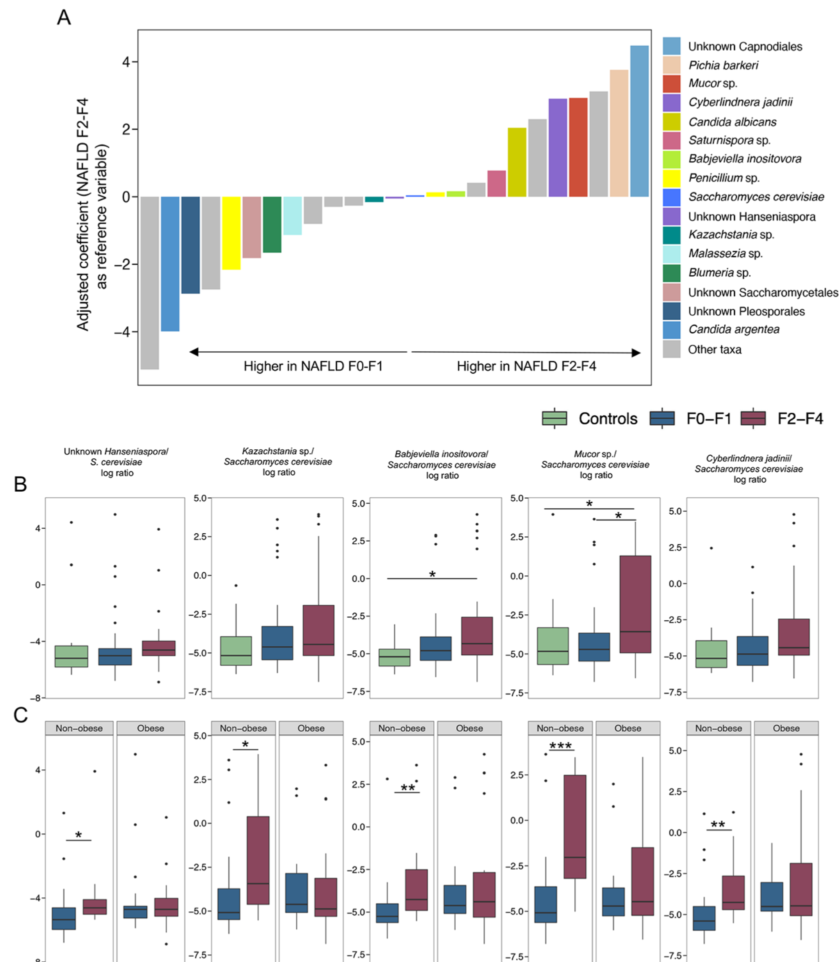

Figure 3. Compositional changes in the fecal mycobiome of patients with F2-F4 fibrosis.

(A) Differential abundance of fungal taxa among groups were calculated using Songbird under consideration of clinical metadata (age, gender, BMI, type 2 diabetes, proton pump inhibitor use and alcohol consumption). Fungal taxa with at least 90% confidence level alignment in the UNITE database were colored. (B) Log-ratios of specific fungal taxa that were significant among groups, either when including all patients, or (C) when investigating subgroups based on obesity. In (A-C), 78 patients with NAFLD were included in the analysis of whom 40 had minimal fibrosis (F0-F1) and 38 had at least moderate fibrosis (F2-F4). Of the patients with F0-F1 fibrosis, 23 (57.5%) were non-obese and 17 (42.5%) were obese. Of the patients with F2-F4 fibrosis, 15 (39.5%) were non-obese and 23 (60.5%) were obese. In (B-C), 16 subjects without any known disease were additionally included. *P* values determined by Kruskal-Wallis test with Dunn's post-hoc test for skewed distributions followed by false discovery rate (FDR) or Whitney-Wilcoxon rank-sum test. $^{*}P<0.05$, >0.01; $^{**}P<0.01$, $P>0.001$; $^{***}P<0.001$. For the box and whisker plots (B-C), the box extends from the 25th to 75th percentile, with the center line indicating the median; the bottom whiskers indicate the minimum values and the top whiskers indicate the 75th percentile plus 1.5-fold the inter-quartile distance (the distance between the 25th and 75th percentiles). Values greater than this are plotted as individual dots.

NAFLD, non-alcoholic fatty liver disease.

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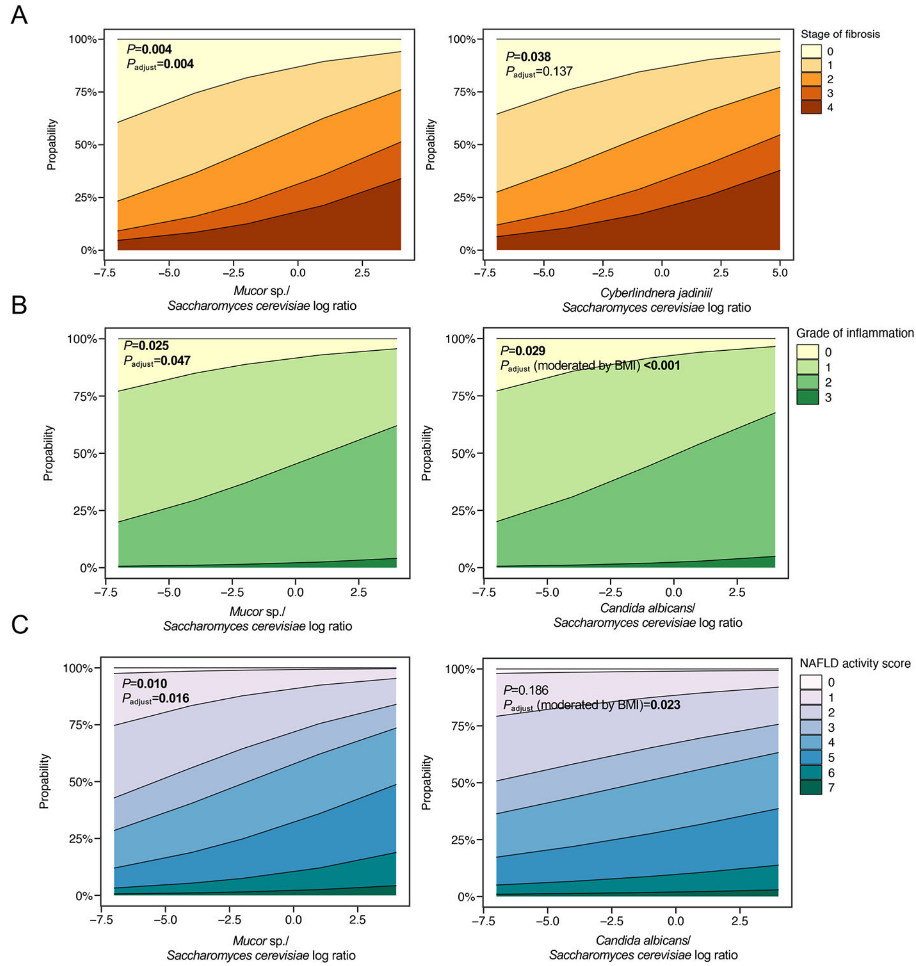

Figure 4. Associations between compositional fecal mycobiota changes and liver histology features.

Univariate and multivariate ordinal regression models using liver histology features as outcome parameter, were used to associate specific log-ratios with liver biopsy features. Predicted probabilities for (A) individual stages of fibrosis, (B) grades of inflammation and (C) the NAFLD activity score. Univariate and multivariate analyses, adjusted for age, gender, BMI, type 2 diabetes, PPI use and alcohol consumption as well as multivariate analyses introducing the BMI as moderating factor, were performed. Only significant ($P<0.05$) associations are shown. In (A-C), 69 biopsy-proven patients with NAFLD were included. Bold font indicates statistical significance ($P<0.05$).

BMI, body mass index; NAFLD, non-alcoholic fatty liver disease.

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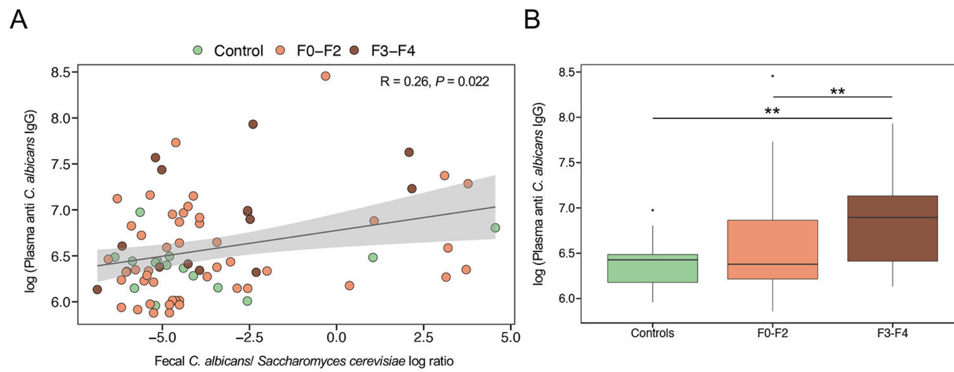

Figure 5. Plasma antibodies against *Candida albicans* are increased in patients with NAFLD and advanced fibrosis.

Antibodies specific to *Candida albicans* (Anti-*C. albicans* IgG) were measured in plasma samples from 96 subjects (17 controls and 79 patients with NAFLD) of whom 74 were overlapping with the subjects for the fecal mycobiome analysis. (A) Spearman correlation between anti-*C. albicans* IgG and *C. albicans*/ *Saccharomyces cerevisiae* log ratio. (B) Anti-*C. albicans* IgG in n=17 controls, n=58 subjects with NAFLD and F0-F2 fibrosis and n=21 subjects with NAFLD and F3-F4 fibrosis. For the box and whisker plot (B), the box extends from the 25th to 75th percentile, with the center line indicating the median; the bottom whiskers indicate the minimum values and the top whiskers indicate the 75th percentile plus 1.5-fold the inter-quartile distance (the distance between the 25th and 75th percentiles). Values greater than this are plotted as individual dots. *P* values determined by Kruskal-Wallis test with Dunn's post-hoc test for skewed distributions followed by false discovery rate (FDR). ***P* <0.01, *P*>0.001.

C. albicans, Candida albicans; Ig, Immunoglobulin; NAFLD, non-alcoholic fatty liver disease

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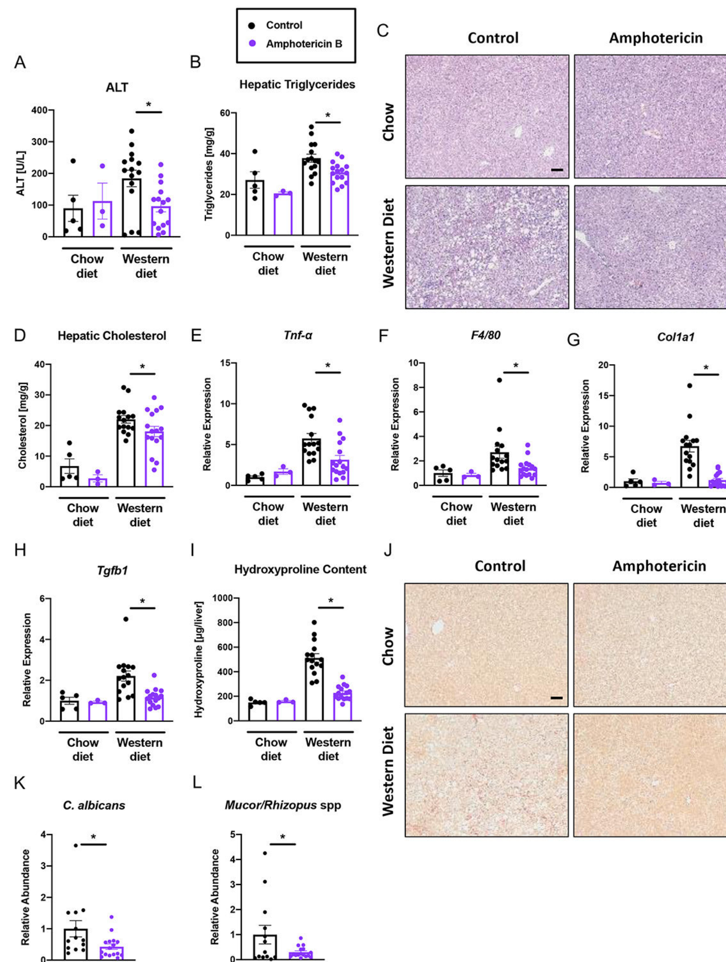

Figure 6. Amphotericin B decreases Western diet-induced steatohepatitis and fibrosis in fecal microbiome-humanized mice.

Germ-free C57BL/6 mice were colonized with feces from two patients with NASH and subjected to 20 weeks of Western diet-feeding with or without amphotericin B (n=13-16 per group), or chow diet with or without amphotericin B (n=3-5 per group). (A) Plasma ALT levels. (B) Hepatic triglyceride content. (C) Representative liver sections after hematoxylin and eosin staining (bar size = 100 μm). (D) Hepatic cholesterol content. (E-H) Hepatic mRNA expression of (E) *Tnf-α*, (F) *F4/80*, (G) *Col1a1*, and (H) *Tgfb1*. (I) Hepatic hydroxyproline content. (J) Representative liver sections after Sirius Red staining (bar size = 100 μm). (K-L) Fecal content of (K) *Candida albicans* and (L) *Mucor/Rhizopus* spp. in Western diet-fed groups. Results are expressed as mean ± s.e.m. *P* values are determined by One-way ANOVA with Holm's post-hoc test (A-I) or unpaired student t-test (K-L). *$P<0.05$. ALT, alanine aminotransferase; Col1a1, collagen type I alpha 1; *Mucor/Rhizopus* spp, *Mucor/Rhizomucor/Rhizopus* species; NASH, non-alcoholic steatohepatitis; Tgfb1, transforming growth factor-beta 1; Tnf-α, tumor necrosis factor-alpha.

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Table 1.

Clinical characteristics of the study cohort

	N/A, n	Controls (n=16)	NAFL (n=24)	NASH (n=54)	*P* value
Age, years		31.7 (7.1)	48.1 (23.2)	58.8 (14.0)	**<0.001**
Gender female, n (%)		10 (62.5)	10 (41.7)	30 (55.6)	0.376
BMI, kg/m^2		20.4 (2.4)	28.7 (5.3)	30.8 (6.0)	**<0.001**
Obesity, n (%)		0 (0.0)	7 (29.2)	33 (61.1)	**<0.001**
Type 2 diabetes, n (%)		0 (0.0)	0 (0.0)	16 (29.6)	**0.001**
Arterial hypertension, n (%)		0 (0.0)	11 (45.8)	40 (74.1)	**<0.001**
Metabolic syndrome, n (%)		0 (0.0)	5 (20.8)	27 (50.0)	**<0.001**
Proton pump inhibitor use, n (%)		0 (0)	1 (4.2)	9 (16.7)	0.081
Waist circumference, cm		83.0 (6.0)	102.0 (18.5)	110.0 (18.8)	**<0.001**
Alcohol consumption, g/d		5.7 (5.8)	1.5 (5.5)	0.0 (2.0)	**0.002**
Albumin, g/L	2	45.0 (1.5)	45.0 (4.0)	44.0 (3.8)	**0.035**
Creatinine, mg/dL	2	0.7 (0.2)	0.8 (0.3)	0.9 (0.3)	0.202
Urea, mg/dL	2	22.0 (13.0)	29.0 (13.5)	29.0 (13.5)	0.177
Uric acid, mg/dL	3	4.0 (0.9)	5.3 (2.1)	6.1 (2.1)	**<0.001**
AST, U/L	1	24.0 (7.2)	30.0 (8.0)	39.5 (29.8)	**<0.001**
ALT, U/L	1	13.0 (12.0)	41.0 (27.0)	52.0 (46.5)	**<0.001**
GGT, U/L	1	14.5 (8.5)	89.0 (106.0)	70.0 (73.5)	**<0.001**
Alkaline phosphatase, U/L	2	56.0 (14.0)	76.0 (31.5)	74.5 (29.5)	**0.002**
Bilirubin, mg/dL	3	0.5 (0.5)	0.6 (0.5)	0.5 (0.4)	0.583
Ferritin, µg/L	4	51.0 (44.5)	178.0 (193.5)	207.0 (179.0)	**<0.001**
Triglycerides, mg/dL	3	64.5 (72.8)	118.0 (91.5)	170.0 (128.5)	**<0.001**
Total cholesterol, mg/dL	3	150.5 (26.8)	181.0 (41.0)	194.5 (50.5)	**0.003**
HDL cholesterol mg/dL	10	64.0 (15.0)	56.0 (20.8)	45.0 (15.0)	**<0.001**
LDL cholesterol mg/dL	13	70.0 (19.0)	111.0 (44.8)	119.0 (44.5)	**<0.001**
Platelet count, x1E9/L	1	240.5 (34.0)	202.0 (110.0)	219.5 (101.5)	0.451
INR	3	1.0 (0.0)	1.0 (0.1)	1.0 (0.1)	0.239
HbA1c, %	21	4.9 (0.2)	5.2 (0.5)	5.5 (1.0)	**0.002**
Fasting glucose, mg/dL	1	82.0 (8.5)	93.0 (8.5)	101.0 (29.8)	**<0.001**

Values are presented as median and interquartile range in parentheses. Groups were compared using one-way ANOVA or the Kruskal-Wallis test for skewed distributions. Categorical variables were compared using the Fisher's exact test. The number of missing values within the overall cohort is indicated in the second column ("N/A"). Bold font indicates statistical significance ($P<0.05$).

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyl-transferase; HbA1c, glycated hemoglobin; INR, international normalized ratio; HDL, high-density lipoprotein; LDL, low-density lipoprotein; N/A, not available.