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Microbiome Signatures Associated with Steatohepatitis and Moderate to Severe Fibrosis in Children With Nonalcoholic Fatty Liver Disease

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Abstract

Background & Aims: The intestinal microbiome might affect development and severity of nonalcoholic fatty liver disease (NAFLD). We analyzed microbiomes of children with and without NAFLD.

Methods: We performed a prospective, observational, cross-sectional study of 87 children (8–17 years old) with biopsy-proven NAFLD and 37 children with obesity without NAFLD (controls). Fecal samples were collected and microbiome composition and functions were assessed using 16S rRNA amplicon sequencing and metagenomic shotgun sequencing. Microbial taxa were identified using zero-inflated negative binomial modeling. Genes contributing to bacterial pathways were identified using gene set enrichment analysis.

Results: Fecal microbiomes of children with NAFLD had lower α-diversity than controls (3.32 vs 3.52; P=.016). Fecal microbiomes from children with nonalcoholic steatohepatitis (NASH) had lowest α-diversity (controls, 3.52; NAFLD, 3.36; borderline NASH, 3.37; NASH 2.97; P=.001). High abundance of *Prevotella copri* was associated with more severe fibrosis (P=.036). Genes for lipopolysaccharide biosynthesis were enriched in microbiomes from children NASH (P<.001). Classification and regression tree model with level of alanine aminotransferase and relative abundance of the lipopolysaccharide pathway gene encoding 3-deoxy-D-manno-octulosonate 8-phosphate-phosphatase identified patients with NASH with an area under the receiver operating characteristic curve value of 0.92. Genes involved in flagellar assembly were enriched in fecal microbiomes of patients with moderate to severe fibrosis (P<.001). Classification and regression tree models based on level of alanine aminotransferase and abundance of genes encoding flagellar biosynthesis protein had good accuracy for identifying cases with moderate to severe fibrosis (area under the receiver operating characteristic curve, 0.87).

Conclusions: In an analysis of fecal microbiomes of children with NAFLD, we associated NAFLD and NASH with intestinal dysbiosis. NAFLD and its severity were associated with greater abundance of genes encoding inflammatory bacterial products. Alterations to the intestinal microbiome might contribute to pathogenesis of NAFLD and be used as markers of disease or severity.

Lay summary:

The intestinal microbiota of children with nonalcoholic liver disease is altered compared to that of children without this chronic liver disease, with increased levels of bacterial proteins that promote inflammation. These microbial features were associated with liver disease severity.

Keywords

intestinal microbiota; pediatric; lipopolysaccharide; flagellin

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in American children.[1] The diagnosis of NAFLD requires that 5% or more hepatocytes exhibit macrovesicular steatosis, and the exclusion of other identifiable liver diseases and/or clinical conditions, which may cause steatosis. A comprehensive understanding of why certain children develop NAFLD is lacking. Although obesity is a risk factor for NAFLD, most children with obesity do not develop NAFLD. A subset of children with NAFLD has a progressive sub-phenotype known as nonalcoholic steatohepatitis (NASH), characterized by hepatic inflammation and cell injury. Moreover, some children with NASH develop cirrhosis and end-stage liver disease. Thus, NAFLD is not a singular diagnosis, but a broad clinical-pathological spectrum of liver disease.[2] Factors that explain the severity of disease are poorly characterized. Emerging evidence suggests that the intestinal microbiome may influence the development and severity of NAFLD.

The intestinal microbiome is a complex ecosystem composed of trillions of microbes, mainly in the large bowel, living in a predominantly symbiotic relationship with the host and broadly impacting host physiology, immune development and function, nutrition, and mucosal protection.[3–5] However, it may also impact human pathophysiology, including inflammatory bowel disease, and obesity[6] by influencing local and systemic inflammation[7,8] and the host capacity for energy harvest.[9,10] Additionally, animal models suggest that it may influence several of the putative processes involved in the development and progression of NAFLD,[11] including choline metabolism, endotoxemia, obesity, liver inflammation and fibrosis.[12–16]

While mouse models have implicated intestinal bacteria in the pathophysiology of NAFLD, [17] evidence in humans is more limited, especially in children. Although some human studies have evaluated the fecal microbiota in children with NAFLD, they have been small and in some cases, limited by the lack of optimally characterized cases and controls.[18,19] Therefore, we evaluated the fecal microbiome of children with NAFLD, and age and Body Mass Index (BMI)-matched control children without NAFLD, characterizing the bacterial composition and functional capacity that discriminate between cases and controls and those that associate with greater disease severity.

METHODS

Study subjects

This was a prospective, observational, cross-sectional ancillary study to the NASH CRN performed at its UC San Diego Clinical Center. Participants were age 8-17 years. Cases were children with NAFLD diagnosed within 90 days using standard clinical history, laboratory, and liver histology criteria.[20] Controls were overweight or obese children recruited from

primary care clinics without evidence of liver disease based on clinical history, labs and liver Magnetic Resonance Spectroscopy (MRS). Use of probiotics or recent (past 90 days) antibiotics exposure was exclusionary. Institutional Review Boards at each participating center approved the study. Parents provided written informed consent and children provided written assent.

Clinical assessment

Demographic and clinical data for each participant was obtained at a single intake visit at the UCSD Altman Clinical and Translational Research Institute. Height and weight were measured, and fasting blood samples were obtained for glucose, insulin and, liver and lipid panels. Fresh fecal samples were collected.

Liver histology

Liver biopsy specimens were stained with hematoxylin-eosin and Masson's trichrome stains, and reviewed centrally by the NASH CRN Pathology Committee according to the published NASH CRN histological scoring system scoring system.[21] The diagnosis of nonalcoholic steatohepatitis (NASH), borderline NASH, or NAFLD not NASH was made based on the aggregate presence and degree of the individual features of nonalcoholic fatty liver disease. Fibrosis was staged based on the following: stage 0 (no fibrosis), stage 1a (mild perisinusoidal), stage 1b (moderate perisinusoidal), stage 1c (portal fibrosis), stage 2 (perisinusoidal and periportal), stage 3 (bridging fibrosis), and stage 4 (cirrhosis).

Liver MRS

Proton Density Fat Fraction (PDFF) was estimated using MRS by a long-TR, multi-TE stimulated echo acquisition mode (STEAM) sequence using established methodology (see supplement for full details).[22,23]

Fecal sample collection and processing

Fresh fecal samples were placed in RNAlater™ and rocked overnight at 4°C and stored at −80°C until isolation. Genomic DNA was isolated from fecal samples using the MO BIO PowerFecal DNA Isolation Kit™ (MO BIO) with following modification: after addition of solution C1 and heating at 65°C for 10 minutes, sample was subjected toheating at 95°C for 10 minutes followed by bead beating using the PowerLyzer™ (MO BIO).

16S rRNA gene amplicon sequencing

Polymerase chain reaction (PCR) amplification was performed using primers targeting V1-V3 region of the bacterial 16S rRNA gene. PCR products were purified using 1.8x volume Agencourt AMPure XP™ beads (Beckman Coulter) and quantified using the Qubit® dsDNA BR assay or dsDNA HS assay (Life Technologies). Samples were multiplexed and sequenced on the Illumina MiSeq™ platform to generate 300bp, paired-end reads. (see supplement for full details).

Metagenomic whole genome shotgun (mWGS) sequencing

mWGS libraries were constructed with 100-250 ng of sample DNA, using KAPA HTP library preparation kits™ (KAPA Biosystems), with 8 (for 250 ng) or 10 (for 100 ng) PCR amplification cycles. Library concentrations were determined using the KAPA qPCR kit™ (KAPA Biosystems). Libraries were multiplexed and run on Illumina HiSeq 2000™ platform to generate 100bp paired-end reads.

Bioinformatic Processing of Sequence Data

Paired-end 16S reads were assembled into contiguous sequences and clustered at a 97% sequence similarity threshold using USEARCH [24] to generate set of 1136 Operational Taxonomic Units (OTUs). These were filtered to retain 712 OTUs, which were either present in >5% of samples, or constituted >1% of the reads in one or more sample.

To quantify relative gene abundance in the intestinal metagenome, mWGS sequences were mapped to curated reference gene database using HUMAnN2.[25] The function of detected genes was determined by aligning them to the Kyoto Encyclopedia of Genes and Genomes (KEGG) reference database (see supplement for full details).[26]

Data Analysis

Patients' characteristics were summarized as median and interquartile range or n (%). A non-parametric Mann-Whitney-Wilcoxon test was used to compare cases and controls while a Kruskal-Wallis test was used to compare *Prevotella copri* low, medium, and high abundance (see supplementary methods) in continuous variables. A Chi-square or Fisher's exact test was performed for categorical variables. To compare continuous variables between groups, a t test or Analysis of Variance (ANOVA) was performed. If parametric assumptions were not met even after transformation (typically log), then a non-parametric method was used. Individuals were classified as low, medium, or high based on relative abundance of *P. copri* (see results and supplementary methods), and distribution of these categories by fibrosis, NASH, or steatosis was examined using a linear-by-linear association exact test. Fibrosis stage was dichotomized into none to mild (stages 0 to 1) or moderate to severe.

Due to the sparseness of the microbiome data, a zero-inflated negative binomial model [27] was applied to test the differential effect of NAFLD or fibrosis on the relative abundance of bacteria at different taxonomic levels. For those that did not converge, a negative binomial or Poisson model was used instead. To account for the potential confounding factor of ethnicity, ethnicity (Hispanic vs Non-Hispanic) was included as a covariate for those genera or OTUs that were significant at $p<0.05$.

Comparisons of microbiome community between individuals (β-diversity) were based on a Bray-Curtis distance matrix of normalized OTU counts and evaluated using principal coordinates analysis in conjunction with analysis of similarity (ANOSIM) tests.[28]

Classification and regression trees (CART) were used to explore relationships between fibrosis, α-diversity and microbial taxon abundance. Fibrosis (absent/mild vs moderate/severe) was treated as the target variable and the following were predictors: *Bacteroides vulgatus* abundance, *P. copri* abundance, and Shannon α-diversity index. The options for

parent node and terminal node were 10 and 5, respectively. Ten percent leave-out samples were used for cross-validation. Optimization was performed using a Gini function.

Differential abundance of microbial gene pathways was tested by gene set enrichment analysis (GSEA). Microbial genes were represented as KEGG Orthologs (KOs, see supplementary methods), and a p-value was generated for each KO using a gamma model or a mixture model. KOs were ranked by p-value and the relative enrichment of KEGG pathways within KO rankings was calculated using the R package piano.[29] Direction of the change in relative abundance of each KO was determined based on the direction (positive vs. negative) of the normalized Wilcoxon signed-rank test statistic. Significantly enriched pathways were identified at $p<0.05$ following False Discovery Rate (FDR) correction. In comparisons of none/mild vs. moderate/severe fibrosis GSEA was carried out separately for high and low *P. copri* individuals. Pathways were reported as significant if they had an FDR-adjusted p-value<0.05 for both groups (for justification of this approach see supplementary methods). CART were also performed to determine important factors in predicting case vs control, NASH vs NAFLD but not NASH, Moderate-to-severe fibrosis vs. absent-to-mild fibrosis using ALT and abundance of KOs from the LPS biosynthetic pathway or the flagellar assembly pathway as predictor variables. Area Under the Receiver Operating Characteristics curve (AUROC) was calculated to evaluate the performance.

The relative contribution of different bacterial genera to microbial gene pathways of interest was assessed using indicator values,[30] which were generated and tested using the R package labdsv (see supplementary methods).[31]

Unless otherwise stated, statistical significance was assigned at $p<0.05$. FDR-adjusted $p<0.2$ was considered significant for comparisons of taxa at different levels. Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC), StatXact (Cytel Studio version 8.0.0. Cytel Inc.), R (version 3.3.2 or 3.4.2, Vienna, Austria), and Salford Predictive Modeler (SPM) software suite CART (Salford Systems, San Diego, CA). R package phyloseq [32] was used and microbial community analysis was performed using the R package Vegan.[33]

RESULTS

Study population

There were 124 children studied, with a median age of 12 years. Demographics and clinical features are shown in Table 1 (Supplementary Fig. 1). Cases (n=87) and controls (n=37) did not differ by age or race. Children with NAFLD were more likely to be male, 71% versus 46% (p=0.0073). Cases and controls were well-matched for BMI and percent body fat.

Taxonomic composition of the intestinal microbiome (full study cohort)

Using 16S rRNA gene sequencing, an average of 67,852 gene sequences were generated per individual and 712 discrete bacterial taxa (OTUs) identified. At the phylum level, dominant taxa consisted of the Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. At the genus level, dominant taxa were *Bacteroides*, *Faecalibacterium*, and *Blautia*, as well as a number of unclassified members of the family *Lachnospiraceae* (Supplementary Fig. 2).

Differences in the composition of the intestinal microbiome between cases and controls

The phyla Bacteroidetes and Proteobacteria were higher in cases (zero-inflated negative binomial model: fold change (FC): 1.34, FDR-adjusted p=0.051 and FC: 1.57, FDR-adjusted p=0.025, respectively), whereas the phylum Firmicutes was higher in controls (FC: 0.84, FDR-adjusted p=0.025). Genus-level comparisons revealed statistically significant differences in the relative abundance of multiple sparse genera, but few differences in dominant taxa (Fig. 1A, Supplementary Table 1). After adjusting for ethnicity, the majority (14/19) of the genera remained significant. In some cases multiple OTUs assigned to the same genus showed opposite trends of increased/decreased relative abundance between cases and controls (Supplementary Fig. 3, Supplementary Table 1), indicating the potential for differences between distinct taxa belonging to a single genus.

At the community level, cases had significantly lower α-diversity than controls (3.32 vs 3.52, t-test: p=0.016, Fig. 1B). Microbiome composition (β-diversity) did not differ between cases and controls (ANOSIM test, p=0.71); however, principal coordinates analysis (PCoA) demonstrated greater person-to-person microbiome variability among cases (Fig. 1C, multivariate homogeneity groups dispersion test: p=0.061). Person-to-person variability in microbiome composition was correlated with relative abundance of OTUs for two genera - *Bacteroides* and *Prevotella*. Species represented by these OTUs were inferred by reconstructing their sequence-based phylogeny (Supplementary Fig. 4, Supplementary Methods) indicating that dominant OTUs likely represented the species *P. copri* and *B. vulgatus*.

Individuals clustered into three discrete groups based on relative abundance of *P. copri* (Supplementary Fig. 5, Supplementary Methods), but did not cluster by *B. vulgatus* relative abundance. The dominance of *B. vulgatus* or *P. copri* within the microbiome appeared mutually exclusive (Supplementary Fig. 5C); however, this trend may not indicate competitive exclusion as suggested previously,[19] but may reflect that with finite sequencing abundance estimates are relative, not absolute. Similarly, greater relative abundance of both *B. vulgatus* and *P. copri* correlated with lower α-diversity (Supplementary Fig. 6). In addition, high *P. copri* abundance was significantly associated with Hispanic ethnicity (p=0.025, Supplementary Table 2).

Association of microbiome composition with disease severity

The phyla Proteobacteria and TM7 were higher in patients with NASH (FC: 1.97, p=0.027), while the phyla Fusobacteria, Verrucomicrobia, and Lentisphaerae were higher in patients with NAFLD but not NASH (FC: 0.0006, FDR-adjusted p=0.0033, FC: 0.013, FDR-adjusted p<0.001, FC: 0.084, and FDR-adjusted p=0.15, respectively). At genus level, *Lactobacillus* and *Oribacterium* were higher in patients with NASH, while *Oscillibacter, Lactonifactor, Akkermansia*, and *Enterococcus* were higher in patients with NAFLD but not NASH (FDR-adjusted p<0.2 Fig. 2A). The genera *Akkermansia, Lactobacillus, Oscillibacter,* and *Parabacteroides* remained significant after adjusting for ethnicity. At OTU level, multiple taxa belonging to the same genus showed opposite trends in abundance (Supplementary Fig. 7).

Community-level changes accompanying NASH were characterized by a significant difference in α-diversity across the spectrum of NAFLD (ANOVA test: p=0.012, Fig. 2B). Patients with NASH had lower α-diversity compared to controls (2.97 vs 3.52, p=0.001), patients with NAFLD but not NASH (2.97 vs 3.36, p=0.017), or patients with borderline NASH (2.97 vs 3.37, p=0.016).

When classified by fibrosis stage (≤1 vs ≥2), the phyla Bacteroidetes (FC: 1.43, FDR-adjusted p=0.091), Proteobacteria (FC: 1.72, FDR-adjusted p=0.062) and TM7 (FC: 3.41, FDR-adjusted p=0.062) were more abundant in patients with moderate-to-severe fibrosis. Fusobacteria (FC: 0.0007, FDR-adjusted p=0.0022), Verrucomicrobia (FC: 0.027, FDR-adjusted p<0.0001), Firmicutes (FC: 0.85, FDR-adjusted p=0.16) were more abundant in patients with absent-to-mild fibrosis. At genus level, the most abundant taxa identified as differentially abundant were *Lactobacillus* (higher in moderate-to-severe fibrosis), *Akkermansia* (higher in absent-to-mild fibrosis, Fig. 3A). *Slackia, Akkermansia, Gemella, Granulicatella, Lactobacillus,* and *Turicibacter* remained significant after adjusting ethnicity. Also, in several cases multiple OTUs assigned to the same genus showed conflicting trends with fibrosis severity (Supplementary Fig. 8).

Comparison across the range from control to NAFLD without or with severe fibrosis showed lower α-diversity (p=0.009, Fig. 3B) and higher frequency of high *P. copri* abundance (p=0.036, Fig. 3C) among those with greater fibrosis. Because *P. copri* abundance was associated with both lower α-diversity and Hispanic ethnicity, CART analysis was performed and showed that BMI and *P. copri* abundance were the best predictors of fibrosis severity (Supplementary Fig. 9) and that ethnicity did not add to the models ability to identify fibrosis severity.

Association of microbiome gene pathways with case status and severity

Differences in the composition of the microbiome (Fig. 1C) correlated strongly with functional differences, as reflected by variation in microbial gene diversity (Mantel R=0.86, p (perm) <0.001, Supplementary Fig. 10A). However, the total number of genes in the metagenome did not vary with presence of NAFLD, or disease severity (Supplementary Figs. 10–11).

Using GSEA, 30 pathways were differentially abundant in one or more pairwise comparisons (Table 2). No evidence was found for variation in abundance of gene pathways associated with alcohol metabolism and this negative result was subsequently corroborated using serum alcohol assays (see supplementary methods). There was significantly greater prevalence of pathways associated with carbohydrate metabolism in the milder disease state for each pairwise comparison (Case vs. control, NASH vs NAFLD but not NASH, Moderate-to-severe fibrosis vs. absent-to-mild fibrosis), while there was significantly greater prevalence of pathways whose products stimulate host inflammation in the more severe disease state for each pairwise comparison.

Lipopolysaccharide (LPS) biosynthesis was significantly enriched in cases (FDR-adjusted p<0.0001, Table 2, Fig. 4A) and further enriched in those with NASH (FDR-adjusted p=0.0135, Table 2, Fig. 4E). CART models using ALT and the abundance of KOs within the

LPS biosynthesis pathway as predictors had good diagnostic accuracy for discriminating NAFLD cases (AUROC=0.95) and for discriminating cases with NAFLD but not NASH from those with definite NASH (AUROC=0.92). Measuring the abundance of a single KO from the LPS pathway was sufficient in each CART model: the relative abundance of genes encoding for 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase (kdsC) provided the best prediction for NAFLD (Fig. 4B,C); while the relative abundance of genes encoding for UDP-3-O-acyl-N-acetylglucosamine deacetylase (lpxC) provided the best prediction for NASH (Fig. 4F,G). Examination of the taxonomic origin of shotgun-sequenced reads indicated that 32 bacterial genera contributed to LPS biosynthesis. Indicator values were used as a concise metric to represent the extent to which the contribution of different genera to this pathway was characteristic of either cases vs. controls, or NAFLD vs. NASH (Fig. 4D,H).

Flagellar assembly was also enriched in cases (FDR-adjusted $p<0.0001$, Table 2, Fig. 5A), and in those with moderate-to-severe fibrosis (FDR-adjusted $p<0.0001$, Fig. 5A, E). CART models based on ALT and the abundance of KOs within the flagellar assembly pathway had good diagnostic accuracy for discriminating NAFLD cases (Fig. 5B,C, AUROC=0.97) and for discriminating cases with absent-to-mild from those with moderate-to-severe fibrosis (Fig. 5F,G, AUROC=0.87). KOs selected for inclusion in CART models predicting NAFLD represented genes encoding motility protein B (MotB), flagellar hook-basal body complex protein (FliE), flagellar basal body P-ring formation protein (FlgA, Fig. 5A,B). The single KO included in CART models for fibrosis represented genes encoding Flagellar biosynthesis protein (FlhA, Fig. 5E,F). There were 16 bacterial genera that contributed to flagellar assembly and the relative contribution of specific taxa to this pathway varied across conditions (Fig. 5D, H). In particular, the genus *Lactobacillus* made a significantly greater contribution to flagellar assembly in cases vs control (FDR-adjusted $p=0.187$) and in moderate/severe fibrosis (FDR-adjusted $p=0.03$).

Examination of the genera contributing to pro-inflammatory gene pathways was additionally carried out on an individual-by-individual basis (Supplementary Figs 12–15). This indicated that a substantial contribution from the genus *Lactobacillus* to flagellar assembly was only observed in a subset of individuals (Supplementary Figs. 14–15). Furthermore, other bacterial taxa appeared to make substantial contributions to these pathways in only one, or few, individuals. This indicated that, while the overall abundance of these pathways associated with the presence and severity of NAFLD, the taxa responsible for each pathway was likely to vary between individuals.

DISCUSSION

We studied the association of intestinal microbiota with NAFLD in well-characterized children with obesity with and without NAFLD. Lower α-diversity of the intestinal microbiome was associated with both the condition of having NAFLD and the severity of NAFLD. High *P. copri* abundance was associated with lower α-diversity and greater fibrosis. Genes for lipopolysaccharide biosynthesis were enriched with NASH and genes for flagellar assembly were enriched with moderate-to-severe fibrosis. Microbial genes were associated

with disease severity and provided for prediction models for NASH and for moderate-to-severe fibrosis with good accuracy.

NAFLD is associated with dysbiosis of the intestinal microbiome

In this study, we demonstrated that pediatric NAFLD was associated with two community-level changes in the intestinal microbiome. The first of these was a decline in α-diversity, which may reflect a reduction in the number of different microbial taxa (i.e. richness), and a bias towards overrepresentation of a smaller number of these taxa. Interestingly, declines in α-diversity were not associated with fewer microbial genes detected, meaning there was no evidence of lower metabolic potential despite lower diversity. This differs from previous studies of adult obesity where loss of diversity[6] was linked to a reduction in metabolic potential.[34]

The second community-level change associated with NAFLD was greater person-to-person variability in composition of the microbiome. While this trend was relatively minor, a reanalysis of microbiome data originating from obese vs. lean twins showed similar increases in variability within obese individuals.[35] This trend is also consistent with recent observations that increased heterogeneity is associated with a variety of stressors in many microbiome-related disorders.[35,36] In contrast to α-diversity, person-to-person variability correlated strongly with the composition of the intestinal metagenome, suggesting that greater heterogeneity within cases was accompanied by differences in microbial metabolic potential.

The influence of *Bacteroides, Prevotella,* and diversity on NAFLD

The genera *Bacteroides* and *Prevotella* are dominant members of the healthy human intestinal microbiome.[4] We classified patients into discrete groups based on a bimodal distribution in the relative abundance of *P. copri,* which was the dominant species belonging to the genus *Prevotella*.

When considering the relative abundance of *Bacteroides* and *Prevotella,* previous studies have found conflicting results. Zhu et al.[37] reported no difference in *Bacteroides* and a significant difference in *Prevotella* relative abundance when comparing children with healthy-weight, obesity, or NASH from Buffalo, NY; *Prevotella* was more abundant in children with NASH or obesity and less abundant in healthy controls.[38] However, this trend was driven by greater *Prevotella* relative abundance between healthy-weight vs. obese/NASH individuals. In a study of 61 Italian children with obesity or NAFLD matched to 54 controls, there was no association between liver disease and *Bacteroides* or the *Prevotella* genus.[18] However, an examination of publically available data suggests that few children with NAFLD in Italy would have been classified as having high *Prevotella* relative abundance. In adults in France, Boursier et al. reported significantly higher *Bacteroides* relative abundance, and lower *Prevotella* relative abundance in patients with NASH.[19] These differences may be attributed in part to both diet and geography, with high *Prevotella* abundance linked to fiber-rich diets and high *Bacteroides* abundance linked to a high protein, Western diet.[39–41] Perhaps varying conclusions in studies from different geographic regions

are due to host factors in that race/ethnicity, genetics, and diet may play a larger role in the relationship between disease and microbiome than is currently known.

A common theme is that lower microbial α-diversity is associated with NAFLD.[18,37,42] As microbial abundance estimates are relative,[43] changes in α-diversity are closely linked to the relative abundance of all taxa. Accordingly, we show that the relative abundance of dominant OTUs from both *Bacteroides* and *Prevotella* correlated inversely with α-diversity. Given this observation, we hypothesize that previously reported increases in the relative abundance of either taxon may be explained by a loss of diversity accompanying NAFLD-associated dysbiosis. The specific dominant taxon showing increased relative abundance because of such dysbiosis may thus depend on whether *Bacteroides* or *Prevotella* dominate the background of the individual in question. Under this hypothesis, it also follows that it may be the loss of diversity, not the increased relative abundance of dominant taxa that is affecting NAFLD.

Alternatively, the setting of decreased diversity may increase the potential for the dominant organisms themselves to impart negative consequences. In our study we report evidence for an association between the genus *Prevotella* and aspects of the NAFLD phenotype. In particular we observed that individuals classified as high *P. copri* were more frequently cases with moderate-to-severe fibrosis. We also demonstrated that it was *P. copri* relative abundance, not α-diversity, which best predicted liver fibrosis. These findings along with the known biology of *P. copri* suggest a potential link between high levels of *P. copri* and fibrosis progression. Previous studies have reported that increased *P. copri* relative abundance may exacerbate arthritis,[44] possibly via TH17 cell-mediated inflammation.[45] Moreover, in contrast to other bacteria present in the intestinal microbiome (e.g. *Bacteroides), P. copri* produces superoxide reductase and phosphoadenosine phosphosulphate reductase. Together, these enzymes grant *P. copri* a higher tolerance to host-derived reactive oxygen species through the conversion of highly toxic superoxide to less toxic hydrogen peroxide, and the production of thioredoxin, thus providing *P. copri* a competitive advantage by allowing it not only to thrive in a proinflammatory environment, but also perhaps increase intestinal inflammation for its own advantage.[46] Such pro-inflammatory effects may therefore exacerbate liver damage during NAFLD progression and are worthy of further investigation.

Therefore, we hypothesize both that a loss in diversity and an increase in the abundance of *P. copri* may be causal factors influencing NAFLD. While these hypotheses are inevitably confounded, they need not be mutually exclusive. Indeed they may each independently be exerting influence on different aspects of the NAFLD disease spectrum.

Pro-inflammatory pathways associated with disease severity

In addition to taxonomic differences, we identified two pro-inflammatory pathways, containing genes encoding for LPS biosynthesis and flagellar assembly, which were present at greater relative abundance in the NAFLD metagenome. LPS biosynthesis genes had greater relative abundance in the metagenome of cases vs. controls and individuals with NASH vs. NAFLD, while flagellar assembly genes had greater relative abundance in the

metagenome of cases vs. controls and individuals with moderate-to-severe fibrosis vs. absent-to-mild fibrosis.

LPS is a potent activator of innate immunity via toll-like receptor TLR4 stimulation in macrophages, neutrophils, and dendritic cells.[47] It is a component of the cell walls of gramnegative bacteria, including the phylum Proteobacteria as well as the genera *Bacteroides* and *Prevotella*. Critically, TLR4 activation has also been shown to stimulate the production of Th17 cells from naïve CD4 T cells,[47] and a Th17 driven pro-inflammatory state is involved in NAFLD pathogenesis. Th17 subsets are elevated in patients with NAFLD and obesity and the Th17 response has been linked with progression to NASH.[48] TLR4 signaling plays a critical role in NAFLD progression as well. TLR4 expression is increased in wild-type mice with steatohepatitis in response to high-fat diet,[15] and TLR4-mutant mice are resistant to NAFLD.[49,50]

Bacterial flagellin is also a potent activator of the innate immune system, both via the toll-like receptor TLR5[51] and via NOD-like receptor-mediated inflammasome activation.[52] TLR5 is expressed in the intestinal epithelium where it plays an important role in exacerbating the symptoms of inflammatory bowel disease. While LPS is a ubiquitous feature of gram-negative bacteria, only a subset of bacteria express flagella. Furthermore, only certain taxa are known to stimulate TLR5.[51] The taxonomic origin of genes detected in this pathway is therefore of interest. However, we caution that overlap between genes encoding for flagellar assembly and type III secretion systems mean further investigation is warranted in order to validate the true function of genes identified as contributing to this pathway.

Multiple bacteria were found to contribute to these pro-inflammatory pathways. Most notably, the genus *Lactobacillus* made a significantly greater relative contribution to flagellar assembly in cases and in individuals with moderate-to-severe fibrosis. This genus also showed the largest increase in relative abundance of any taxon identified as significant in comparisons of both NAFLD vs. NASH and absent-to-mild vs. moderate-to-severe fibrosis. Motility in Lactobacillus appears restricted to a small number of species.[53] While some of these species possess adaptations that limit their pro-inflammatory effects,[54] broad conservation of the TLR5 interaction motif suggests that many are capable of triggering a TLR5-mediated immune response.[53]

Implication of *Lactobacillus* via both 16S and shotgun approaches makes this genus of particular interest. However, another prominent feature of our analysis was that a variety of taxa made substantial contributions to pro-inflammatory pathways in a small number of individuals. These taxa may therefore also be relevant to the etiology of NAFLD; however, their taxonomic diversity, sparse occurrence, and low global abundance of may have prevented their detection in this, and other, 16S based studies. Many of these taxa belong to the phylum Proteobacteria, which we observed at greater abundance in NAFLD cases. Similarly, Zhu et al. reported greater abundance of Proteobacteria in patients with NASH.[37] This phylum may be particularly permissive for opportunistic pathogens that can influence NAFLD.

There have been recent advances using bacterial taxonomy to predict NAFLD disease states[55] however evidence of functional redundancy between different microbial taxa linked to pro-inflammatory pathways suggests that genes, rather than taxa are likely to prove better biomarkers for the identification and characterization of NAFLD. Accordingly, we found that genus abundance measurements were inadequate for predicting disease using CART. In contrast, accounting for the abundance of genes within the LPS biosynthesis and flagellar assembly pathways resulted in a significant improvement over predictive models based on ALT alone.

The current study was notable for the large sample size of well-characterized children with NAFLD including liver histology evaluated in a standardized fashion by expert liver pathologists blinded to the clinical and laboratory details. In addition, the study design maximized the ability of the controls to serve as a proper comparison group. These children were recruited from the same local communities as the cases and absence of liver disease was proven with history, physical, labs, and MRS-PDFF. However, factors that can influence the microbiome such as diet were not evaluated. Notably, our study population was predominantly of Hispanic ethnicity and this was controlled for in our analyses. We encourage further elucidation of the relationship between ethnicity and the microbiome in the context of NAFLD and this will require studies that consider both cultural and biological factors. Finally, the cross-sectional design allowed for association but not causation. Mechanistic links could be evaluated through longitudinal studies in patients, and in targeted translational studies in gnotobiotic and/or genetically modified animal models.

Conclusions

We examined the intestinal microbiome in a well-characterized pediatric cohort with and without NAFLD. We identified significant differences in the NAFLD intestinal microbial community characterized by lower α-diversity and greater variation in β-diversity. Collectively, we refer to these changes as NAFLD-associated dysbiosis. We further conclude that NAFLD-associated dysbiosis is characterized by changes in the functional capacity of the intestinal microbiome, including greater abundance of genes encoding for the pro-inflammatory bacterial products LPS and flagellin. CART models using microbial gene abundance as predictors support the potential for the microbiome to be a clinically useful biomarker. Multiple bacteria were found to contribute to these pro-inflammatory pathways, with distinct groups of contributing taxa in each individual. These findings may explain the conflicting findings of prior studies, while suggesting essential biological roles for the microbiome in NAFLD pathophysiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NAFLD	Nonalcoholic Fatty Liver Disease
NASH	Nonalcoholic Steatohepatitis
NASH CRN	NASH Clinical Research Network
BMI	Body Mass Index
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
HDL	High Density Lipoprotein
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
GGT	Gamma Glutamyl Transpeptidase
STEAM	Stimulated Echo Acquisition Mode
PDFF	Proton Density Fat Fraction
AMARES	Advanced Method for Accurate, Robust and Efficient Spectral
PCR	Polymerase Chain Reaction
ANOVA	Analysis of Variance
ANOSIM	Analysis of Similarity
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG Orthologs
OTU	Operational Taxonomic Unit
FDR	False Discovery Rate
CART	Classification and Regression Tree
PCoA	Principal Coordinate Analysis
GSEA	Gene Set Enrichment Analysis
LPS	Lipopolysaccharide
TLR	Toll-Like Receptor
mWGS	Whole Metagenome Shotgun

UniProt	Universal Protein Resource
SPM	Salford Predictive Modeler

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What you need to know:

BACKGROUND AND CONTEXT:

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in children in the United States. Alterations to the intestinal microbiome might affect development or severity of NAFLD.

NEW FINDINGS:

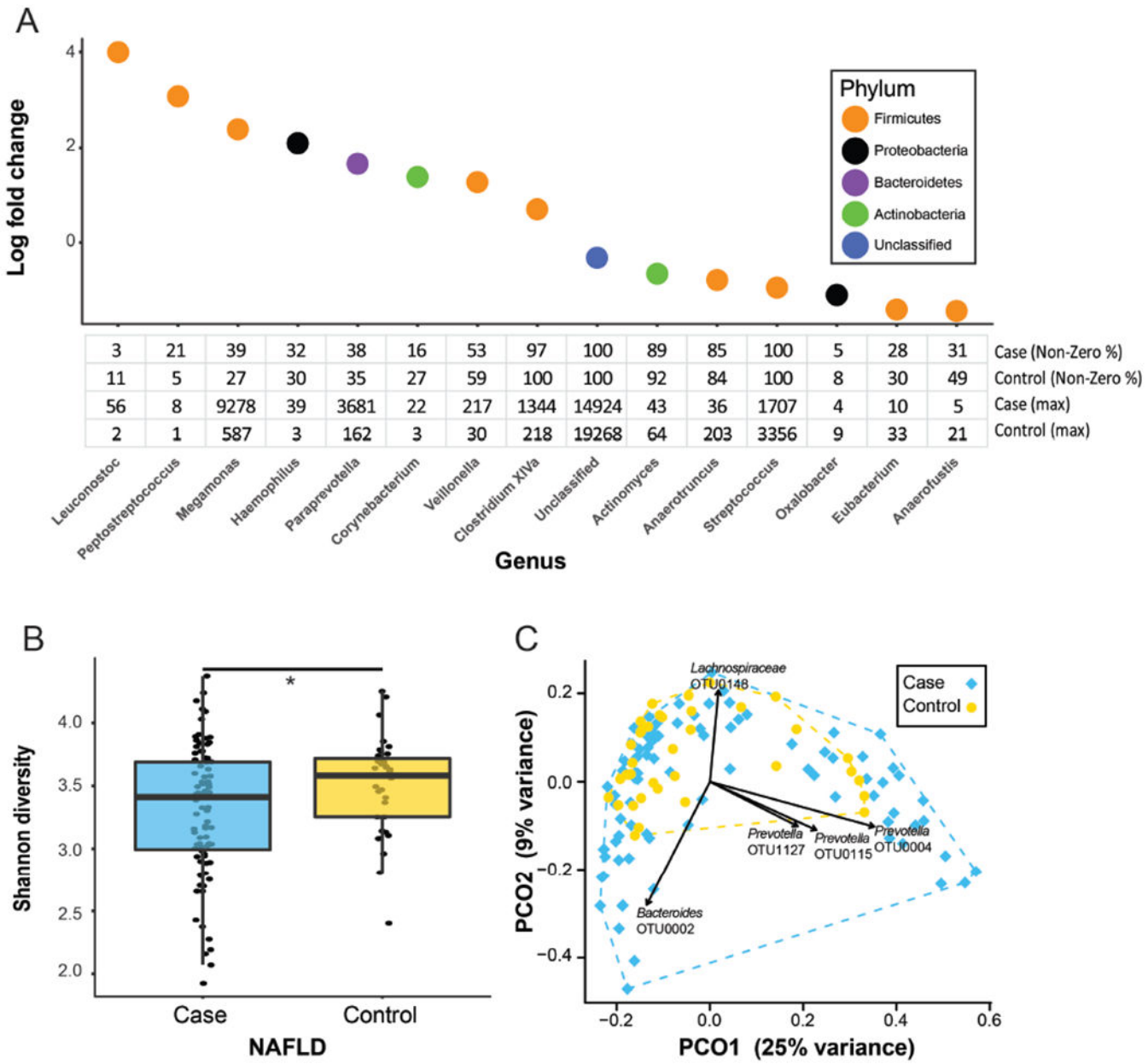
We found fecal microbiomes of children with NAFLD to be disrupted compared to children without NAFLD. Increased levels of bacterial genes that regulate synthesis of lipopolysaccharide assembly of flagella were associated with disease severity and identified children with steatohepatitis or moderate to severe fibrosis.

LIMITATIONS:

This was a cross-sectional study of associations; the findings should be replicated in other cohorts. The mechanisms proposed should be assessed in animal models.

IMPACT:

Characterization of fecal microbiomes and genes expressed by intestinal microbes might provide insights into the pathogenesis of NAFLD in children and lead to discovery of therapeutic targets and diagnosis markers.



3	21	39	32	38	16	53	97	100	89	85	100	5	28	31
11	5	27	30	35	27	59	100	100	92	84	100	8	30	49
56	8	9278	39	3681	22	217	1344	14924	43	36	1707	4	10	5
2	1	587	3	162	3	30	218	19268	64	203	3356	9	33	21

Figure 1. Taxonomic and community-level changes between NAFLD versus controls.

(A) Bacterial genera identified as being differentially abundant when comparing cases to controls and accounting for ethnicity. The table shows percentage of individuals with non-zero read counts for each genus and maximum number of reads detected in any single individual following normalization. (B) Difference in α-diversity between cases and controls (*$p<0.05$). (C) Principal coordinates analysis (PCoA) plot showing variation in β-diversity between cases and controls. Each point represents a single patient. Overlaid vectors indicate the direction of change in abundance for top five OTUs correlating with distribution of individuals within the plot.

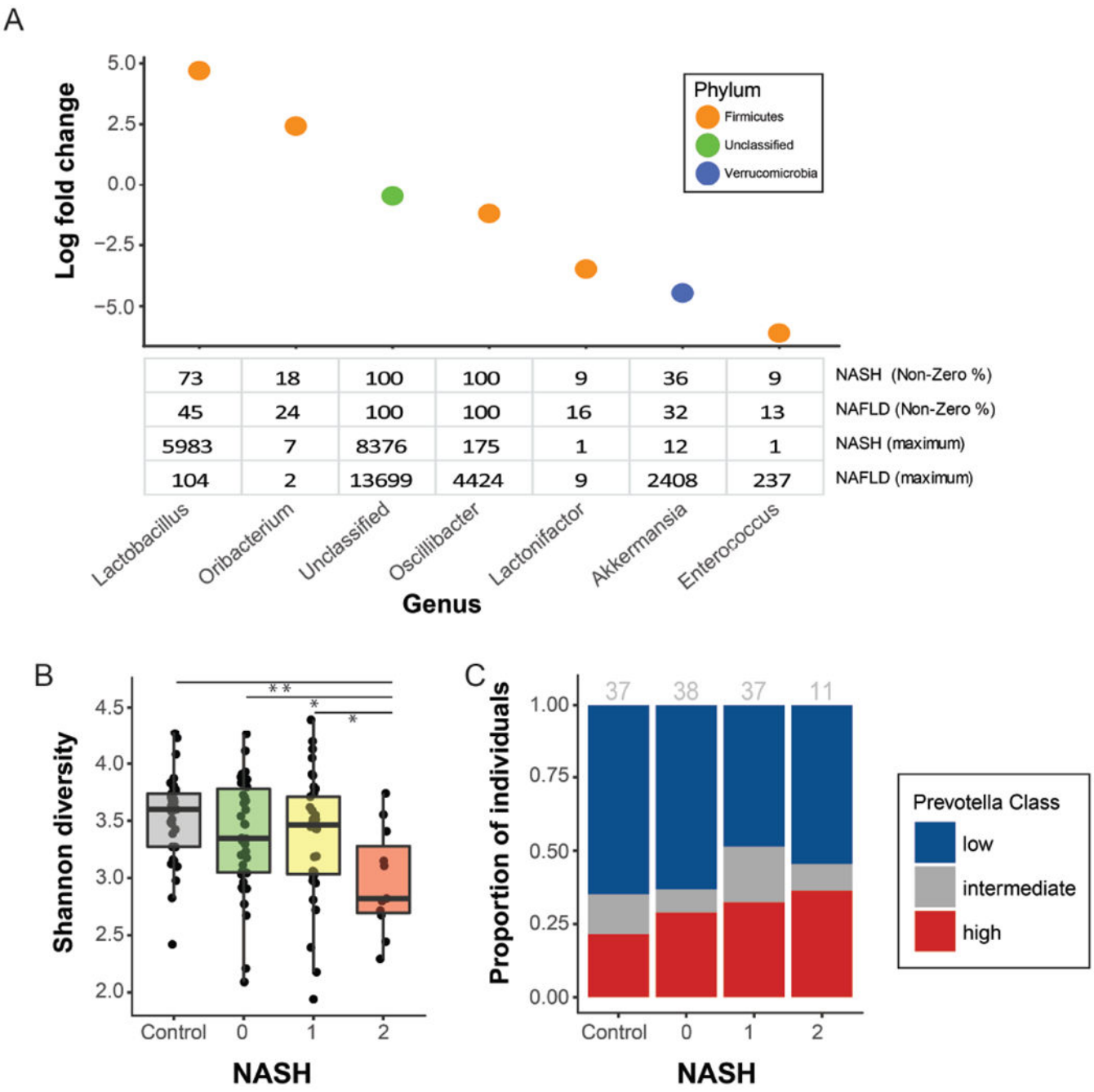


Figure 2. Taxonomic and community-level changes between NAFLD vs. NASH.

(A) Bacterial genera identified as differentially abundant comparing cases with NAFLD but not NASH vs. definite NASH when accounting for ethnicity. The table shows percentage of individuals with non-zero read counts for each genus and the maximum reads detected in any single individual following normalization. (B) Difference in α-diversity between controls and cases separated by severity (control, 0 = NAFLD but not NASH, 1 = borderline NASH, 2 = definite NASH). (C) Distribution of *Prevotella* abundance categories (low, intermediate, high,) by severity (control, 0 = NAFLD but not NASH, 1 = borderline NASH, 2 = definite NASH); number per group shown in gray.low, intermediate, or high *Prevotella*

intestinal microbiome by severity (control, 0 = NAFLD but not NASH, 1 = borderline NASH, 2 = definite NASH). Gray numbers above each bar indicate the number of individuals per group.

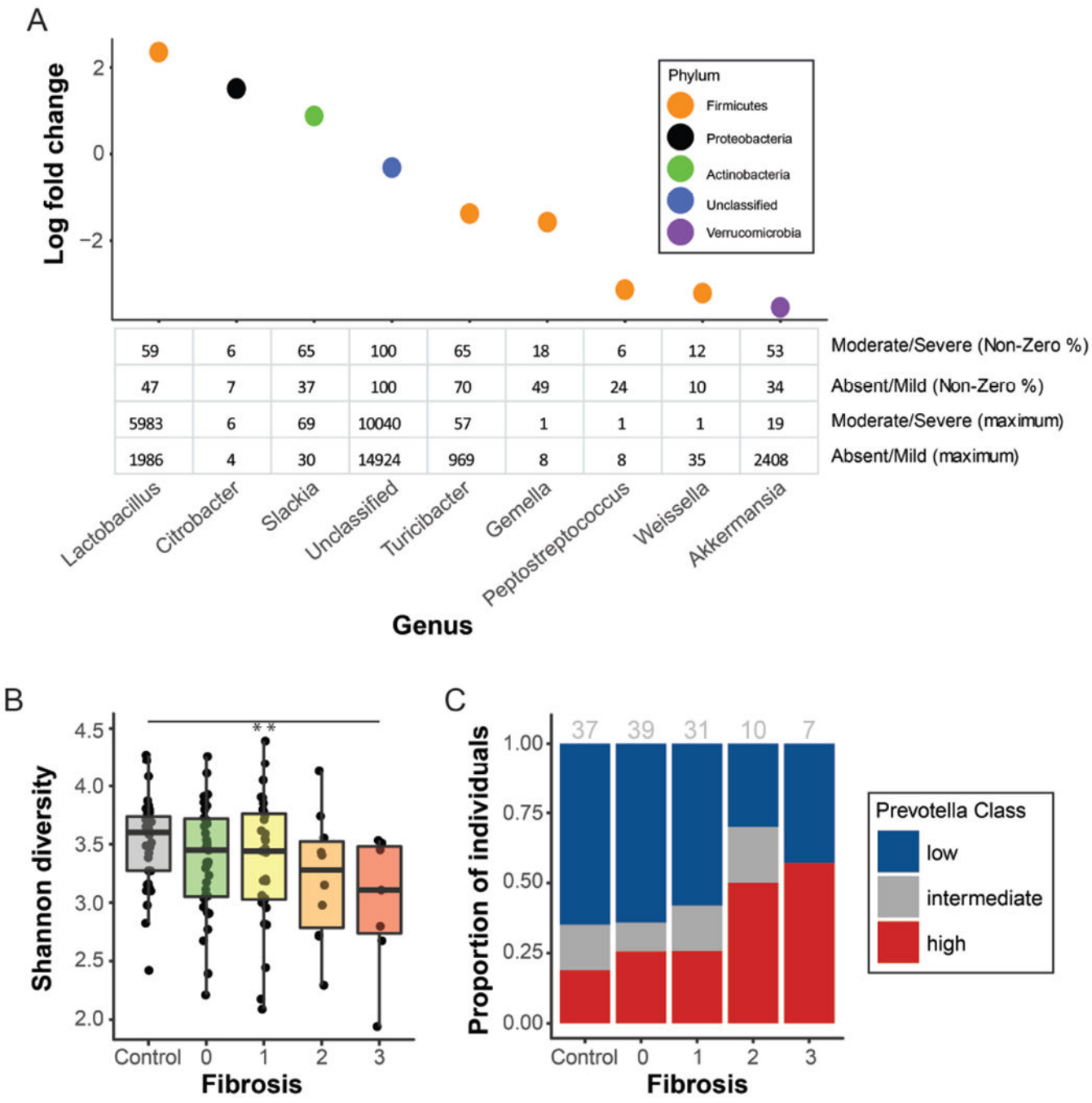


Figure 3. Taxonomic and community-level changes between absent-to-mild vs. moderate-to-severe-fibrosis.

(A) Bacterial genera differentially abundant by fibrosis stage (≤1 vs ≥2) when accounting for ethnicity. Table shows percentage of individuals with non-zero read counts for each genus and maximum reads detected in any single individual following normalization. (B) Difference in α-diversity across fibrosis stages (control, NAFLD with stages 1,2,3) (C) Distribution of *Prevotella* abundance categories (low, intermediate, high) across across fibrosis stages (control, NAFLD with stages 1,2,3); number per group shown in gray.

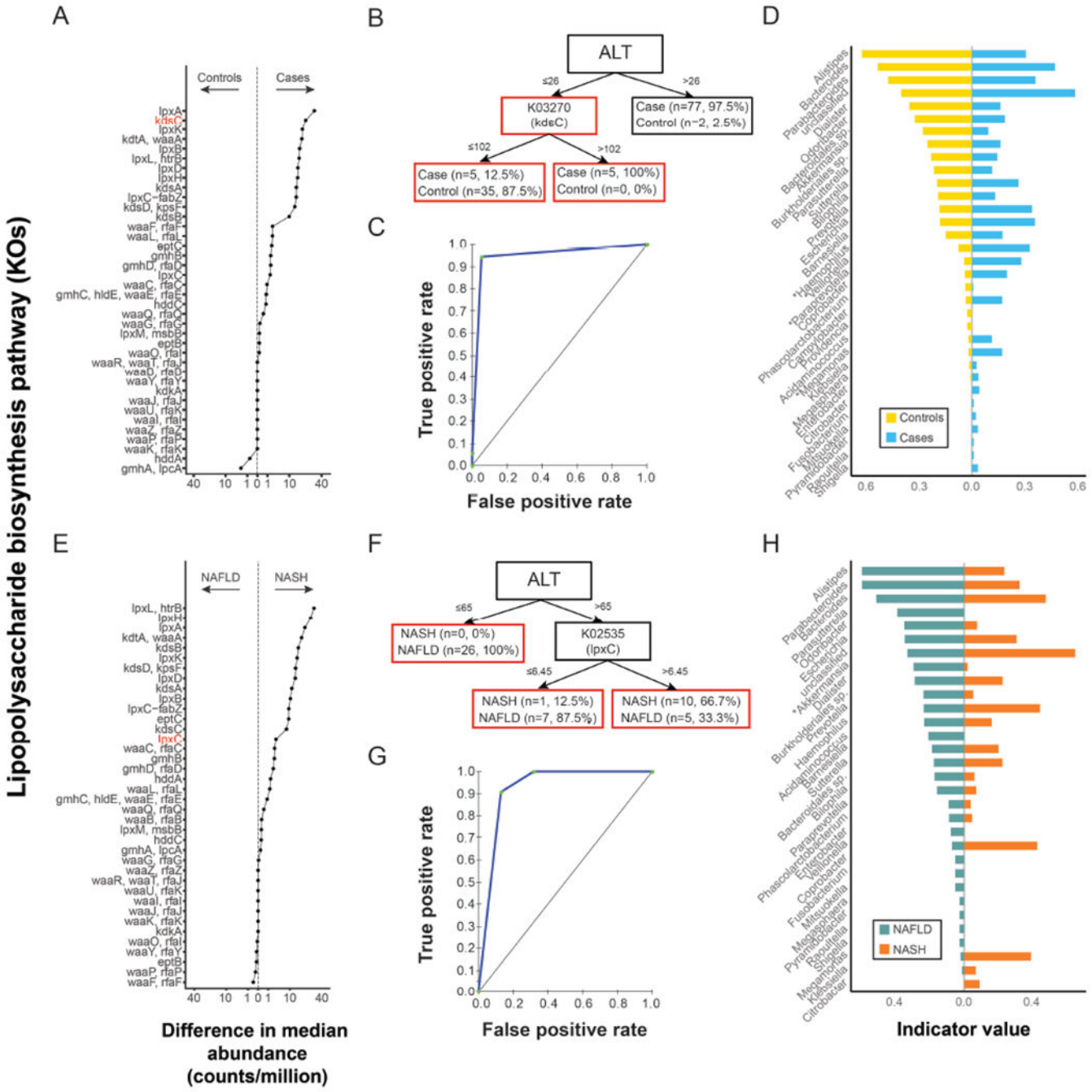


Figure 4. Lipopolysaccharide biosynthesis (LPS) pathway association with NAFLD and NASH

(A) KEGG Orthologs (KOs) in LPS pathway shown on y-axis and the absolute difference (controls vs cases) in median relative abundance of each KO shown on the x-axis. Values to the left and right of the dashed line indicate KOs found at greater relative abundance in controls and cases, respectively. KOs in red are those appearing in panel B. **(B)** Classification and regression tree (CART) model for NAFLD based on ALT and the abundance of KOs from the LPS biosynthesis pathway . The corresponding ROC is shown in panel **(C)**. **(D)** Bacterial genera contributing to LPS assembly in controls and cases are

shown on the y-axis. The x-axis depicts indicator values. A large indicator value indicates a genus with both high fidelity to a condition (i.e. the genus was detected as contributing to this pathway in a large proportion of individuals with that condition) and high specificity to a condition (i.e. the genus was found to contribute more to the condition in question than to the alternative condition in the contrast). Asterisks indicate genera present in Fig. 1A. **(E)** Difference in the median number of reads mapping to the LPS pathway in patients with NAFLD vs NASH. The x and y axis are as described for A. **(F)** CART model for detecting NASH based on ALT and the abundance of KOs from the LPS pathway as predictor variables. The corresponding ROC is shown in panel **(G)**. **(H)** Bacterial genera contributing to LPS assembly in patients with NAFLD and patients with NASH. The x and y axis are as described for D. Asterisks indicate genera present in Fig. 2A.

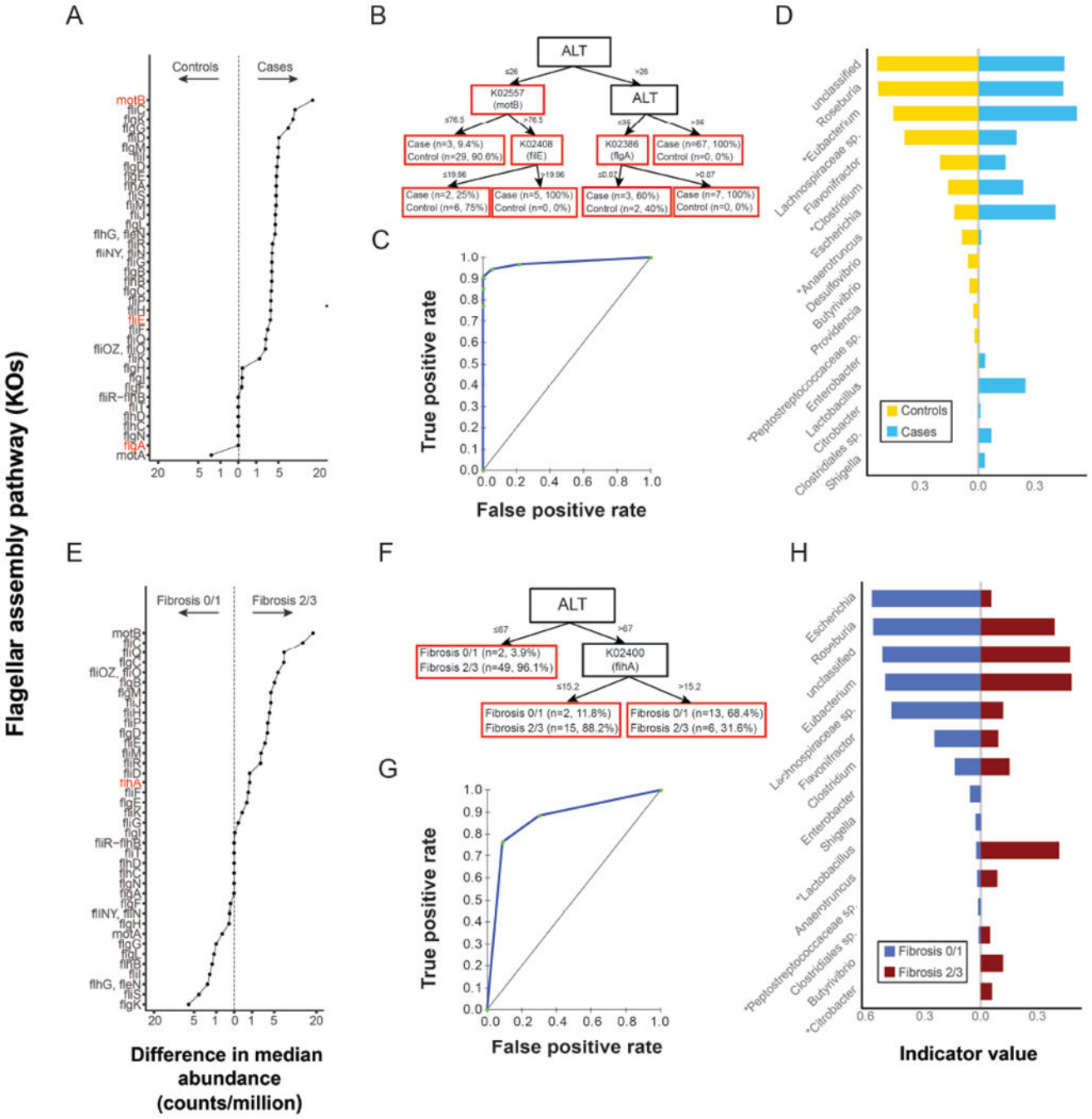


Figure 5. Flagellar assembly pathway associations with NAFLD and with fibrosis severity

(A) KEGG Orthologs (KOs) in the flagellar assembly pathway shown on y-axis and the absolute difference (controls vs cases) in median relative abundance of each KO shown on the x-axis. Values to the left and right of the dashed line indicate KOs found at greater relative abundance in controls and cases, respectively. KOs in red are those appearing in panel B. **(B)** CART model for NAFLD based on ALT and the abundance of KOs from the flagellar assembly pathway as predictor variables. The corresponding ROC is shown in panel **(C)**. **(D)** Bacterial taxa genera contributing to flagellar assembly in controls and cases are

shown on the y-axis. The x-axis depicts indicator values. Asterisks indicate genera present in Fig. 1A. **(E)** difference in the median number of reads mapping to the flagellar assembly pathway in patients by fibrosis stage (≤1 vs ≥2) . The x and y axis are as described for A. **(F)** CART model for moderate-to-severe fibrosis based on ALT and abundance of KOs from the the flagellar assembly pathway as predictor variables. The corresponding ROC is shown in panel **(G)**. **(H)** Bacterial genera contributing to flagellar assembly in patients by fibrosis stage (≤1 vs ≥2). The x and y axis are as described for D. Asterisks indicate genera present in Fig. 3A.

Table 1.

Patient Characteristics: Case vs Control

	Case (n=87)	Control (n=37)	P value
Gender			
Male	62(71)	17(46)	0.0073[a]
Ethnic			
Hispanic	77(89)	28(76)	0.070
Race			
White	39(45)	13(35)	0.32[a]
Age	12(10,14)	13(11,14)	0.20[b]
Height (cm)	160(150,167)	157(152,165)	0.62[b]
Weight (kg)	74(61,93)	71(62,84)	0.41[b]
BMI	29(26,34)	29(25,31)	0.35[b]
BMI percentile[c]	99(98,99)	98(95,99)	0.022[b]
BMI Z score[c]	2(2,2)	2(1,2)	0.022[b]
ALT	57(36,100)	16(12,20)	<.0001[b]
AST	40(27,56)	20(18,25)	<.0001[b]
GGT	30(22,45)	18(15,21)	<.0001[b]
Insulin	28(18,41)	23(14,39)	0.21[b]
Glucose	85(79,89)	85(82,89)	0.41[b]
MRS Liver Fat	19(12,24)	4(2,5)	<.0001[b]
Body Fat	42(39,46)	41(37,46)	0.34[b]
Triglyceride	126(90,196)	86(77,121)	0.0073[b]

	Case (n=87)	Control (n=37)	P value
Total cholesterol	164(141,186)	154(135,172)	0.11[b]
HDL cholesterol	42(36,49)	44(39,50)	0.17[b]
LDL cholesterol	94(76,111)	83(72,95)	0.11[b]
Steatosis			
5-33%	28(32)		
34-66%	25(29)		
>66%	34(39)		
NASH Diagnosis			
NAFLD, not NASH	38(44)		
Borderline NASH	37(43)		
Definite NASH	11(13)		
Fibrosis			
Stage 0	39(45)		
Stage 1a	4(5)		
Stage 1b	2(2)		
Stage 1c	25(29)		
Stage 2	10(11)		
Stage 3	7(8)		

Data are presented as median (interquartile range) or n (%).

[a] Chi-Square test;

[b] Mann-Whitney-Wilcoxon test;

[c] Calculated based on the 2000 Centers for Disease Control and Prevention (CDC) growth charts

Table 2

Metabolic pathways enriched in different stages of NAFLD.

Gene set enrichment analysis (GSEA) results showing metabolic pathways that are enriched in comparisons of i) cases (NAFLD+NASH) vs. obese controls, ii) NAFLD (steatosis 0) vs. NASH (steatosis 2), iii) non-severe (fibrosis <2) vs. severe (fibrosis 2+) fibrosis.

Enriched in	Pathway Description	KEGG ID	KOs In Pathway	KOs Detected In Study	GSEA Statistic	p-value	q-value	KOs Up In Rank	KOs Down In Rank
Controls									
	Glycolysis / Gluconeogenesis	ko00010	94	62	0.43754	1.00E-04	0.00355	37	25
	Pentose phosphate pathway	ko00030	75	53	0.47768	0.0041	0.041586	30	23
	Starch and sucrose metabolism	ko00500	101	64	0.32807	0	0	48	16
	Amino sugar and nucleotide sugar metabolism	ko00520	144	94	0.46333	1.00E-04	0.00355	57	37
	Glucagon signaling pathway	ko04922	55	7	0.13289	6.00E-04	0.00852	7	0
	Lysine degradation	ko00310	71	22	0.37371	0.0019	0.022483	15	7
	D-Alanine metabolism	ko00473	5	5	0.12772	0.0021	0.022938	5	0
	Peptidoglycan biosynthesis	ko00550	40	34	0.39466	6.00E-04	0.00852	21	13
	Tetracycline biosynthesis	ko00253	20	6	0.15449	0.0014	0.018073	6	0
	beta-Lactam resistance	ko01501	99	37	0.41112	6.00E-04	0.00852	23	14
	Vancomycin resistance	ko01502	21	17	0.27211	2.00E-04	0.0047333	13	4
	ABC transporters	ko02010	451	321	0.55299	5.00E-04	0.00852	148	173
	Phosphotransferase system (PTS)	ko02060	83	67	0.38192	0	0	47	20
	Aminoacyl-tRNA biosynthesis	ko00970	64	35	0.3799	2.00E-04	0.0047333	21	14
Cases									
	Lipopolysaccharide biosynthesis	ko00540	38	37	0.10385	0	0	4	33
	Riboflavin metabolism	ko00740	26	16	0.055318	1.00E-04	0.00355	0	16
	Bacterial chemotaxis	ko02030	26	26	0.087381	0	0	1	25
	Flagellar assembly	ko02040	41	38	0.053467	0	0	1	37
NAFLD									
	Methane Metabolism	ko00680	158	103	0.26365	1.00E-04	0.0044996	27	76
	Carbon Metabolism	ko01200	342	203	0.43357	1.00E-04	0.0044996	89	114
	Ribosome biogenesis in eukaryotes	ko03008	82	11	0.18249	0.00049995	0.016873	2	9

Enriched in	Pathway Description	KEGG ID	KOs In Pathway	KOs Detected In Study	GSEA Statistic	p-value	q-value	KOs Up In Rank	KOs Down In Rank
	RNA Transport	ko03013	138	12	0.10798	1.00E-04	0.0044996	1	11
	RNA Polymerase	ko03020	51	15	0.24024	0.0009999	0.026997	4	11
NASH									
	Phenylalanine metabolism	ko00360	75	38	0.25417	0.00019998	0.0089991	30	8
	Benzoate degradation	ko00362	90	29	0.26513	0.00069993	0.015748	23	6
	Lipopolysaccharide biosynthesis	ko00540	38	37	0.27594	0.00049995	0.013499	31	6
	Folate biosynthesis	ko00790	44	27	0.27806	0.0017998	0.034711	22	5
	Degradation of aromatic compounds	ko01220	198	38	0.17672	1.00E-04	0.0067493	34	4
	Ribosome	ko03010	143	73	0.33263	0.00039996	0.013499	52	21
	Staphylococcus aureus infection	ko05150	66	7	0.014195	1.00E-04	0.0067493	7	0
Absent/Mild Fibrosis									
	Phosphotransferase system (PTS)	ko02060	83	64	0.31372	0	0	16	48
				65	0.34107	0	0	18	47
	Fructose and mannose metabolism	ko00051	96	70	0.42412	5.00E-04	0.0095714	27	43
				66	0.40166	2.00E-04	0.0132	27	39
Moderate/ Severe Fibrosis									
	Flagellar assembly	ko02040	41	37	0.21208	0	0	31	6
				37	0.25479	0.0004	0.0132	33	4
	Ribosome	ko03010	143	84	0.15512	0	0	81	3
				60	0.14784	0	0	54	6