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Changes in the gut microbiome associated with liver stiffness improvement in nonalcoholic steatohepatitis

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Abstract

Background: Longitudinal studies are needed to decipher mechanistic links between the gut microbiome and nonalcoholic steatohepatitis (NASH). We examined shifts in the gut microbiome in persons with NASH with improvement in liver stiffness measurement (LSM) by magnetic resonance (MR) elastography.

Methods: Gut microbial profiling was performed at baseline and study completion (24 weeks) using 16 S rRNA gene sequencing in 69 adults with biopsy-confirmed NASH and significant fibrosis (stages 2–3) enrolled in a multi-center randomized controlled trial evaluating selonsertib alone or in combination with simtuzumab. Differential abundance of bacterial taxa at baseline and end of study were examined in participants with and without longitudinal improvement in LSM. Gut microbial shifts that correlated with secondary outcomes, including reduction in MR imaging-derived proton density fat faction (MRI-PDFF) and histologic fibrosis regression were evaluated. Fecal samples from 32 healthy adults were profiled and genus-level multidimensional scaling was used to determine if microbial shifts in persons with NASH improvement represented a shift toward a healthy gut microbiome.

Results: Shifts in abundance of 36 bacterial taxa including *Lactobacillus* ($log2_{FC} = -4.51$, FDR < 0.001), *Enterococcus* ($log2_{FC} = -6.72$, FDR < 0.001), and *Megasphaera* ($log2_{FC} = 7.74$, FDR < 0.001) were associated with improvement in LSM. Improvement in LSM was associated with microbial shifts toward healthy reference ($p = 0.05$). Significant shifts in 10 and 12 bacterial taxa were associated with improvement in LSM in addition to MRI-PDFF and fibrosis regression, respectively, indicating consistent taxonomic changes across multiple clinical endpoints.

Conclusion: Longitudinal changes in the gut microbiota are observed in adults with NASH and clinical improvement and represent a shift toward a healthy microbiome.

Keywords: biomarker, fibrosis, longitudinal, microbiome, nonalcoholic steatohepatitis

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Introduction

Nonalcoholic fatty liver disease (NAFLD), a hepatic manifestation of metabolic syndrome, has become the leading cause of chronic liver disease worldwide.[1,2] Nonalcoholic steatohepatitis (NASH), a progressive and severe form of NAFLD, is associated with increased risk for cirrhosis, hepatocellular carcinoma, and liver-related death,[3–5] representing a major public health burden. Effective therapeutic interventions for NASH, and in particular NASH-related fibrosis, are sorely needed.

Emerging evidence relates NASH to the composition and function of the gut microbiome,[6] a collection of microorganisms residing in the human

intestine that serves as an integral component of maintenance of health. However, our current evidence is comprised predominantly of cross-sectional studies linking gut dysbiosis with NASH, and as a result, we have limited understanding of exact mechanistic links between the gut microbiota and NASH pathogenesis. Moreover, little is known about whether longitudinal changes in the gut microbiota coincide with changes in NASH disease severity. An understanding of whether gut microbial shifts occur with clinical improvement in NASH is needed to gain insight into causal microbiota-host interactions in disease pathogenesis, expanding our knowledge beyond only cross-sectional correlations.

In this study, we prospectively examined gut microbial shifts in adults with NASH and stage 2–3 fibrosis enrolled in a clinical trial and evaluated microbial shifts in those with and without clinical improvement in liver stiffness as assessed by magnetic resonance elastography. We also examined microbial shifts during the study period associated with secondary clinical outcomes including improvement in magnetic resonance imaging (MRI)-estimated proton density fat fraction as well as histologic measures including fibrosis regression and improvement in hepatic collagen content. Finally, we performed microbiota profiling in a healthy reference cohort in order evaluate whether changes in taxonomic composition in adults with clinical improvement in NASH were reflective of a shift toward a healthy microbiome.

Methods

Patient population

Patients included in this study were enrolled in a multicenter, open-label, phase-2 trial investigating the safety and efficacy of selonsertib (SEL), a selective inhibitor of apoptosis signal-regulating kinase 1 (formerly GS-4997), in combination with simtuzumab (SIM), a humanized monoclonal antibody directed against lysl oxidase-like 2 (formerly GS-6624).[7] The trial was prospectively registered in Clinicaltrials.gov (NCT02466516). Study enrollment criteria and design have previously been described and are summarized in Supplemental Figure 1.[7] Briefly, adults (18–70 years of age) were enrolled at 23 sites in the United States and Canada from June 2015 to March 2016. To be eligible, patients were required to have a liver biopsy within 3 months of screening consistent with a diagnosis of NASH (defined by NAFLD Activity Score (NAS) ≥ 5 with a score of at least 1 for each of three components including steatosis, hepatocellular ballooning, and lobular inflammation) and stage 2 or 3 fibrosis, according to the NASH Clinical Research Network (CRN) Histologic Scoring System.[8] In addition, all patients had at least three of the following features of the metabolic syndrome: abdominal obesity, hypertension, elevated fasting glucose, elevated levels of serum triglycerides, or low levels of high-density lipoprotein cholesterol. During the trial, patients were randomized 2:2:1 to receive treatment with low-dose SEL 6 mg $\pm$ SIM, high-dose SEL 18 mg $\pm$ SIM, or SIM alone. Selonsertib was administered orally once daily and simtuzumab was administered as weekly subcutaneous injections. Randomization was stratified by the presence or absence of type-2 diabetes mellitus. All patients underwent magnetic resonance imaging and elastography (MRI and MRE), liver biopsy, and fecal sample collection at baseline and 24 weeks.

To enable comparisons with the healthy gut microbiome, we included 32 fecal samples from healthy adults participating in the Human Microbiome Project.[9]

Human stool collection, extraction, and sequencing of the 16 S rRNA amplicon

Fecal samples collected from enrolled patients at baseline and week 24 (end of study) were placed in sterile collection containers and immediately stored at −70°C. DNA from fecal samples was extracted with a Mo BIO PowerMag DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) and the variable region 4 of the 16 S rRNA gene was amplified by polymerase chain reaction (PCR) and sequenced in the MiSeq platform (Illumina, San Diego, CA, USA) via the 2×250-bp paired-end protocol and genus-level assignments were made from operational taxonomic unit (OTU) data.

16 S rRNA data processing and analysis

16 S rRNA sequencing data were processed using qiime2 suite of tools.[10] Briefly, raw fastq data were first imported and de-multiplexed prior to removing primers with qiime2 modules. Sequences were further denoised with DADA2

and summary statistics – including base quality, sequencing depth per sample and feature frequency – were performed to assess sequencing quality. Closed reference mapping and subsequent clustering was performed using *vsearch* module. Phyloseq[11] and DESeq2[12] packages were used to manipulate microbiome data and perform differential abundance analysis for clinical parameters of interest.

Longitudinal clinical assessment of NASH

Improvement in liver stiffness, as measured by two-dimensional MRE, was the primary outcome measure in our analyses and defined a priori. Secondary outcomes included improvement in proton density fat fraction (MRI-PDFF) and histologic outcomes of fibrosis regression and reduction in hepatic collagen content.

MRI was performed at baseline and week 24 (end of study) including two-dimensional MRE and proton density fat fraction (MRI-PDFF) assessments. All sites underwent a quality-assessment process prior to study initiation. Assessments were performed by an experienced central reader (University of California San Diego Radiology Reading Center) blinded to treatment assignments and clinical and histologic data.[7] Improvement in liver stiffness was defined as reduction in liver stiffness measurement (LSM, kPa) of 15% or more.[13] Improvement in LSM of 15% or more was a clinical endpoint in the multicenter trial and has been shown in prior studies to have high specificity for a $\geqslant$1 stage reduction in hepatic fibrosis.[13] Improvement in MRI-PDFF was defined as a reduction of 30% or more.[13,14]

Liver biopsies performed at baseline and week 24 were read by a single reader blinded to treatment assignment but not biopsy sequence with fibrosis staging according to the NASH Clinical Research Network classification.[8] Fibrosis regression was defined by reduction in fibrosis score by at least 1 stage combined with a decrease in alanine aminotransferase (ALT) level and improvement in the NAFLD activity score (NAS) and its individual components. Computer-assisted morphometry was used to quantify hepatic collagen content using picrosirius red-stained liver sections. Improvement in hepatic collagen content was defined by a decrease of 30% or more from baseline to end of study.

Statistical analysis

Categorical parameters were compared using chi-square or Fisher's exact tests. Tests of differential microbiome abundance were performed using DESeq2 package with p values linear models adjusted using the false discovery rate (FDR) correction. Differences between patient populations were analyzed using Kruskal–Wallis or Mann–Whitney U tests (two-sided). To explore whether any previously unrecognized properties impact patient population structure and our downstream analyses, we performed Multidimensional Scaling across all timepoints to evaluate patient clustering and potential drivers of these clusters. Correlation between continuous variables was examined with Spearman's Rank test.

This study was approved by the University of California, San Diego Institutional Review Board and the relevant ethics committees of participating study centers, and all participants provided written informed consent for the randomized phase-2 trial (NCT02466516). All authors had access to the study data and reviewed and approved the final manuscript. The reporting of this study conforms to the STROBE statement.[15]

Results

Patient characteristics

Of the 72 patients with biopsy-proven NASH and stage 2 or 3 fibrosis who were randomized and treated during the clinical trial, 69 had fecal samples at baseline and end of study (week 24). Baseline demographic, clinical, and histologic parameters of the 69 adults who were included in this study are summarized in Table 1. The median age was 55 years (interquartile range (IQR) 47–61 years), and 70% of patients were female. The median body mass index (BMI) was 33.1 kg/m^2 (IQR 29.4–38.0 kg/m^2), and 71% of patients included in this study had type-2 diabetes mellitus. The baseline median liver stiffness by MRE was 3.7 kPa (IQR 3.1–4.7 kPa). Twenty-four (35%) and 45 (65%) patients had fibrosis stage 2 (F2) and 3 (F3), respectively. There was no significant change in weight, HOMA-IR, or hemoglobin A1c% in participants during the 24-week study period.[7]

The mean age and BMI of healthy volunteers from the Human Microbiome Project were 26 years and 24 kg/m^2, respectively.

Table 1. Baseline demographic and clinical characteristics of the nonalcoholic steatohepatitis cohort ($n = 69$).

Characteristic		
Age (years)		55 (47–61)
Female sex		48 (70)
Body mass index (kg/m^2)		33.1 (29.4–38.0)
Type-2 diabetes mellitus		49 (71)
White race		63 (91)
Medication use	Statin*	20 (29)
	Insulin	9 (13)
	Metformin	41 (59)
Lipids	Triglycerides, mg/dL	165 (137–210)
	Total cholesterol, mg/dL	189 (167–214)
	High-density lipoprotein, mg/dL	42 (33–49)
	Low-density lipoprotein, mg/dL	122 (100–139)
Serum biochemical levels	Alanine aminotransferase, U/L	59 (47–91)
	Aspartate aminotransferase, U/L	50 (38–74)
	Alkaline phosphatase, U/L	87 (65–103)
	Total bilirubin, mg/dL	0.5 (0.3–0.6)
	Gamma-glutamyl transferase, U/L	54 (42–86)
Magnetic resonance imaging	MRE, kPa	3.7 (3.1–4.7)
	MRI-PDFF, %	16.9 (11.3–21.6)
Liver histologic findings	NAS $\geqslant 6$	50 (72)
	Steatosis grade 2–3	18 (26)
	Lobular inflammation grade 3	48 (70)
	Hepatocyte ballooning grade 2	59 (86)
	Fibrosis stage 2, F2	24 (35)
	Fibrosis stage 3, F3	45 (65)

Values are either *n*(%) or median (interquartile range).
*Statins included: atorvastatin, pravastatin, rosuvastatin, and simvastatin.

Microbial compositional shifts in NASH cohort based on treatment arm

Longitudinal samples from 69 NASH patients yielded on average 29,516 reads. We first examined differentially abundant taxa between baseline and week 24 in the NASH cohort based on treatment group. No taxa were found to be differentially abundant between baseline and end of treatment in the low dose (6 mg) SEL treatment arm. There were 20 differentially abundant taxa in the high-dose (18 mg) SEL treatment arm at end of study when compared to baseline, which

included *Haemophilus parainfluenzae* and Streptophyta (FDR < 0.01) (Supplemental Figure 2). There were 10 and 8 differentially abundant taxa in the low-dose SEL and SIM and high-dose SEL and SIM treatment arms, respectively (Supplemental Figure 2). However, we found that there was no taxonomic overlap between differentially abundant taxa in those participants receiving high-dose SEL monotherapy and those receiving high-dose SEL and simtuzumab, indicating inconsistent bacterial taxonomic shifts across SEL treatment arms. (Supplemental Table 1).

We examined whether longitudinal microbiome change with SEL resulted in qualitative shifts either toward or away from a healthy microbiome composition. As expected, at the phylum level, the NASH patient cohort differed significantly from healthy reference. While the gut microbiome from healthy reference was dominated by Bacteroidetes (67%), Firmicutes (75%) was the most abundant phylum among the NASH cohort. In addition, Proteobacteria, Actinobacteria, and Verrucomicrobia constituted nearly 20% of bacterial taxa in persons with NASH, while the healthy cohort contained less than 7% of these phyla combined. At the genus level, several taxa were differentially abundant in the NASH cohort as compared to healthy reference. While *Blautia*, *Escherichia*, *Dorea*, *Streptococcus*, and *Bifidobacterium* were enriched in persons with NASH as compared to a healthy reference, *Ruminococcaceae* and *Parabacteroides* were significantly less abundant in the NASH cohort at baseline (all $p < 0.001$).

Utilizing a multidimensional scaling method, we calculated the microbiome distance between each NASH patient at baseline and week 24 and the healthy reference cohort. We found a dosage-dependent trend toward a healthy equilibrium, but this was marginally non-significant ($p = 0.07$, one-sided Jonkheere-Terpstra, Supplemental Figure 3).

Microbial compositional shifts associated with longitudinal NASH clinical improvement

We subsequently focused our analysis on examining changes in microbiota composition during the 24-week study period based on whether participants experienced clinical improvement in NASH. Sixty participants had LSM with MRE at baseline and end of study. During the study period, 12 (12/60, 20%) participants had reduction in LSM $\geq 15\%$, as measured by MRE. We identified significant shifts in abundance of 36 bacterial taxa (FDR < 0.05) that were associated with reduction in LSM over the 24-week period, as compared to baseline (Table 2). *Pasteurellales* ($\log2_{FC} = 4.580$, FDR < 0.001), *Lactobacillus* ($\log2_{FC} = -4.511$, FDR < 0.001), *Enterococcus* ($\log2_{FC} = -6.720$, FDR < 0.001), *Megasphaera* ($\log2_{FC} = 7.738$, FDR < 0.001), *Bacteroides eggerthii* ($\log2_{FC} = 5.875$, FDR < 0.001) were among the taxa that shifted significantly in abundance (end of study (24 weeks) *versus* baseline) in those participants with improvement in LSM during the study period (Figure 1).

Changes in microbiota composition during the 24-week study period were also noted in those participants with clinical improvement in NASH based on secondary clinical outcomes. All participants had a baseline and end-of-study liver biopsy for histologic interpretation; 65 participants (94%) had baseline and end-of-study MRI-PDFF assessment. Eleven (11/65, 17%) participants had improvement in MRI-PDFF, 21 (21/69, 30%) experienced fibrosis regression, and 16 (16/69, 23%) had reduction in hepatic collagen content. Significant shifts in abundance of 44, 69, and 46 bacterial taxa (end of study *versus* baseline) were associated with improvement in MRI-PDFF, fibrosis regression, and reduction in hepatic collagen content, respectively (FDR < 0.05, Supplemental Figure 4). We examined for overlap in differentially abundant bacterial taxa associated with both improvement in LSM with MRE and secondary clinical outcomes. This revealed overlap between differentially abundant taxa (end of study (24 weeks) *versus* baseline, FDR < 0.05) across multiple clinical measures of NASH improvement (Table 3). Significant shifts in abundance of 10 bacterial taxa were associated with improvement in both LSM and MRI-PDFF in adults with NASH, including *Lactobacillus*, *Lachnobacterium*, *Sutterella*, *Alcaligenaceae*, and *Burkholderiales* (Supplemental Table 2). Significant shifts in abundance of 12 bacterial taxa (FDR < 0.05) were associated with improvement in LSM and fibrosis regression including *Megasphaera*, *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Bacteroides eggerthi*, and *Bacteroides ovatus* (Supplemental Table 2).

Next, we performed predictive functional profiling of pathway abundances and compared

Table 2. Differentially abundant bacterial taxa (end of study (24 weeks) *versus* baseline) associated with longitudinal reduction in liver stiffness measurement (LSM) by magnetic resonance elastography in adults with nonalcoholic steatohepatitis ($n = 12$).

Taxa	Log_2-fold change	Adjusted *p* value
p__Firmicutes; c__Bacilli	−2.98	0.0003
p__Proteobacteria; c__Betaproteobacteria	3.69	<0.0001
p__Actinobacteria; c__Actinobacteria;o__Actinomycetales	−2.11	0.04
p__Firmicutes; c__Bacilli;o__Bacillales	−1.81	0.02
p__Firmicutes; c__Bacilli;o__Lactobacillales	−3.04	0.0004
p__Proteobacteria; c__Betaproteobacteria;o__Burkholderiales	3.70	<0.0001
p__Proteobacteria; c__Gammaproteobacteria;o__Pasteurellales	4.58	<0.0001
c__Bacilli; o__Lactobacillales;f__Enterococcaceae	−6.72	<0.0001
c__Bacilli; o__Lactobacillales;f__Lactobacillaceae	−4.52	<0.0001
c__Betaproteobacteria; o__Burkholderiales;f__Alcaligenaceae	3.91	<0.0001
c__Gammaproteobacteria; o__Pasteurellales;f__Pasteurellaceae	4.58	<0.0001
o__Bacteroidales; f__Rikenellaceae;g__Alistipes	3.02	<0.0001
o__Lactobacillales; f__Enterococcaceae;g__Enterococcus	−6.72	<0.0001
o__Lactobacillales; f__Lactobacillaceae;g__Lactobacillus	−4.51	<0.0001
o__Lactobacillales; f__Streptococcaceae;g__Lactococcus	−2.28	0.02
o__Clostridiales; f__Lachnospiraceae;g__Lachnobacterium	2.30	0.002
o__Clostridiales; f__Veillonellaceae;g__Acidaminococcus	4.15	<0.0001
o__Clostridiales; f__Veillonellaceae;g__Megasphaera	7.74	<0.0001
o__Burkholderiales; f__Alcaligenaceae;g__Sutterella	3.91	<0.0001
o__Pasteurellales; f__Pasteurellaceae;g__Haemophilus	4.58	<0.0001
f__Micrococcaceae; g__Rothia;s__mucilaginosa	−2.26	0.03
f__Bacteroidaceae; g__Bacteroides;s__eggerthii	5.79	<0.0001
f__Bacteroidaceae; g__Bacteroides;s__ovatus	3.38	0.0008
f__Prevotellaceae; g__Prevotella;s__	−2.04	0.03
f__Rikenellaceae; g__Alistipes;s__indistinctus	3.02	<0.0001
f__Enterococcaceae; g__Enterococcus;s__	−6.72	<0.0001
f__Lactobacillaceae; g__Lactobacillus;s__	−4.74	<0.0001
f__Lactobacillaceae; g__Lactobacillus;s__zeae	−1.81	0.04

(Continued)

Table 2. (Continued)

Taxa	Log_2-fold change	Adjusted *p* value
f__Streptococcaceae; g__Lactococcus;s__	−2.28	0.02
f__Clostridiaceae; g__Clostridium;s_perfringens	2.14	0.04
f__Lachnospiraceae; g__Lachnobacterium;s__	2.30	0.002
f__Veillonellaceae; g__Acidaminococcus;s__	4.15	<0.0001
f__Veillonellaceae; g__Megasphaera;s__	7.74	<0.0001
f__Alcaligenaceae; g__Sutterella;s__	3.91	<0.0001
f__Desulfovibrionaceae; g__Desulfovibrio;s__D168	2.61	0.001
f__Pasteurellaceae; g__Haemophilus;s__parainfluenzae	4.58	<0.0001

Only differentially abundant taxa with adjusted *p* value < 0.05 are shown.

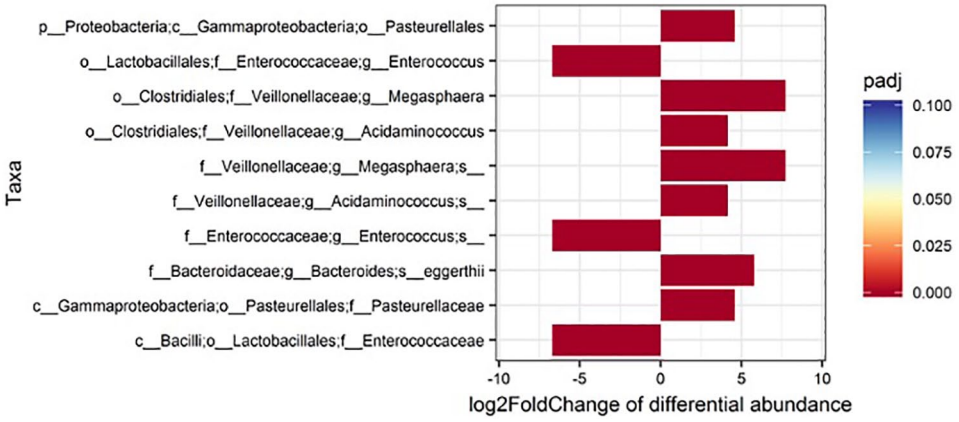

Figure 1. Log2-fold change in the relative abundance of OTUs at baseline as compared to end of study (24 weeks) in patients with NASH and reduction in liver stiffness measurement (LSM), as measured by magnetic resonance elastography ($n = 12$). Top 10 of the total 36 bacterial taxa that were found to be significantly (FDR < 0.05) differentially abundant between LSM progression extremes are displayed.

Table 3. Overlap between significantly differentially abundant taxa (end of study (24 weeks) *versus* baseline, FDR < 0.05) associated with clinical endpoints in adults with nonalcoholic steatohepatitis.

	Reduction in liver stiffness measurement (MRE, kPa)	Reduction in MRI-PDFF	Fibrosis regression	Reduction in hepatic collagen content
Reduction in liver stiffness measurement (MRE, kPa)	36	10	12	9
Reduction in MRI-PDFF	10	44	9	7
Fibrosis regression	12	9	69	13
Reduction in hepatic collagen content	9	7	13	46

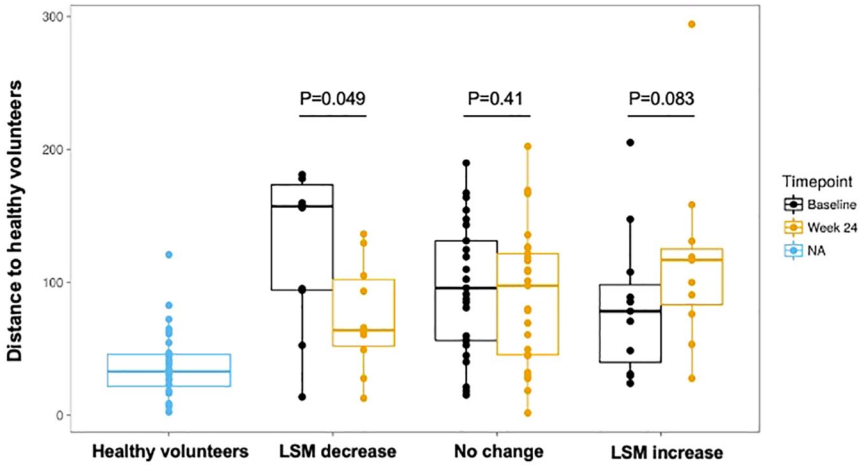

Figure 2. Change in mean difference to healthy reference ($n = 32$) determined by genus-level multidimensional scaling, stratified by change in liver stiffness measurement (LSM) during study period. Change in LSM was categorized as decreased ($n = 12$), no change ($n = 40$), or increased ($n = 8$) during the 24-week study period. Change in LSM was categorized as decreased ($n = 12$), no change (n = 40), or increased ($n = 8$) during the 24-week study period. Decrease in LSM was defined by ⩾15% reduction and indicates clinical improvement in NASH; increase in LSM was defined by a ⩾15% increase. Microbial compositional changes in those participants with NASH and reduction in LSM during the study period represented a shift toward a healthy gut microbiome.

pathway abundance between patients with and without improvement in LSM during the study period. Fecal microbiota in adults with longitudinal improvement in LSM had higher abundance of pathways involved in amino acid metabolism, primary and secondary bile acid biosynthesis, as well as fatty acid biosynthesis, when compared to clinical non-responders (Supplemental Figure 5).

Finally, we evaluated whether microbial compositional shifts in patients with improvement in LSM represented an overall shift toward a healthy microbiome. We observed a striking pattern between the microbiome profile change and change in LSM by MRE. Patients experiencing improvement in LSM (>15% reduction in LSM by MRE) during the 24-week study period shifted their overall microbiome composition toward that of the healthy reference ($p = 0.05$, Figure 2). Intriguingly, persons with improvement in LSM during the study period had a higher distance from healthy reference at study baseline compared to LSM non-responders. On the contrary, patients experiencing ⩾15% increase in LSM by MRE displayed a shift away from the healthy reference, although this did not reach statistical significance, potentially related to the study's small sample size. ($p = 0.08$)

Discussion

A number of recent cross-sectional studies have expanded our current understanding of the gut microbiome in NAFLD,[16] but longitudinal data are limited and needed to better derive mechanistic insights. In this study, we prospectively examined longitudinal changes in the gut microbiota over a 24-week period in adults with biopsy-proven NASH and significant hepatic fibrosis enrolled in an international, multi-center trial. We compared shifts in the gut microbial composition between those with and without clinical improvement in NASH based on advanced MR imaging techniques and histology. In addition, our study incorporated characterization of the gut microbiota in a group of healthy adults for reference, allowing us to determine whether gut microbial dynamics in those with longitudinal improvement in NASH severity were reflective of a shift toward a healthy gut microbiome reference or not.

Our primary analyses were focused on understanding compositional shifts in patients with clinical improvement during the 24-week study period, given there was minimal taxonomic overlap among treatment arms indicating no consistent microbiome associations with treatment, including no taxa

that were differentially abundant at end of study in those participants receiving low dose SEL. We suspect that the lack of taxonomic overlap between treatment arms could be related to recent findings that SIM and SEL do not lead to fibrosis regression in patients with NASH and advanced fibrosis.[7,17] MRE-based assessment of liver stiffness was chosen as the primary outcome given MRE is quantitative, objective, and changes in LSM are likely less affected by sampling error over a 24-week study period.[13,18] Liver stiffness by MRE has also been shown to predict fibrosis progression and risk for hepatic decompensation in NAFLD.[19,20] When examining microbial shifts in persons with improvement in NASH severity as defined by reduction in LSM by 15% or more with MRE, we found significant shifts in 36 bacterial taxa were associated with longitudinal clinical improvement. Moreover, taxonomic shifts in those adults with NASH clinical improvement (decrease in LSM) represented a shift toward a healthy gut microbiome, based on a healthy reference cohort. We also found significant overlap in bacterial taxa shifts that associated with other measures of clinical improvement in NASH, including histologic findings of fibrosis regression and reduction in hepatic collagen content as well as reduction in MRI-PDFF, further strengthening our findings of correlations between gut microbial shifts and NASH clinical improvement.

A number of the bacterial taxa noted to significantly change in abundance in those with clinical improvement in NASH in this cohort have been reported to be associated with NASH histologic phenotypes in cross-sectional analyses, including *Enterococcus spp*, *Veillonella*, *Alistipes*, and *Suturella*.[16] *Bacteroides*, a predominant Gram-negative anaerobe in the gut, significantly increased from baseline to end of study in those persons with improvement in NASH. Intriguingly, certain strains of *Bacteroides ovatus* were recently noted to be necessary for induction of intestinal immunoglobulin A, which has ensuing effects on mitigating bacterial translocation and neutralizing bacterial toxins at the intestinal mucosal surface.[21] Additional strains of *Bacteroides*, specifically *Bacteroides vulgatus*, have been noted to be predominant in NASH-related advanced fibrosis. *Veillonellaceae*, commensal gram-negative cocci that have previously shown to be enriched in cirrhosis and also associated with fibrosis severity in non-obese adults with NASH,[22,23] also shifted during the study period in persons with longitudinal improvement in NASH. Notably, *Veillonella* was recently found to be enriched in adults with NASH and indicative of clinical response to treatment with an analog of the gut hormone fibroblast growth factor 19 (FGF19), likely in part related to bile acid interactions.[24] Emerging evidence has also suggested a role for *Veillonella* in the pathogenesis of inflammatory bowel disease.[25] *Alistipes*, which increased in participants with clinical improvement based on both LSM with MRE and reduction in hepatic collagen content, has been found to be lower abundance in NASH as well as in cirrhosis.[26–28] Moreover, a recent study revealed increase in *Alistipes* that correlated with administration of a glycine-related compound which facilitated NASH improvement in a mouse model.[29] *Megasphaera*, a Gram-negative bacteria in *Veillonellaceae* family and short chain fatty acid producer, has previously been associated with advanced fibrosis in NAFLD.[30] Intringuingly, we noted that *Megasphaera* abundance increased in participants with longitudinal reduction in LSM but decreased in those with histologic fibrosis regression. These findings underscore that microbiome-disease associations are more nuanced than direct associations with microbial abundance. Altogether, the finding of taxonomic shifts that associate with longitudinal improvement in NASH provides insight into microbiota that may be important in the pathogenesis of NAFLD and could be further evaluated for mechanistic links in disease development.

Surprisingly, although we found associations between bacterial taxa and clinical response, we noted marginal shifts in bacterial function based on our predictive functional analysis of bacterial communities. However, it is worth noting that we did find shifts in amino acid metabolism pathways in participants with NASH clinical improvement. Emerging evidence has suggested associations between dysregulated aromatic and branched-chain amino acid metabolism and gut microbiota in NAFLD, leading to interest in the potential for amino acid-related treatments. Plasma concentrations of amino acids, in particular branched-chain amino acids, are increased in NAFLD.[31,32] Moreover, both treatment with microbial-derived products of amino acid metabolism and targeting glutaminolysis (e.g. inhibition of hepatic glutaminase 1 (GLS1)) have been shown to modulate NAFLD in animal models.[29,33–35] Shifts were also noted in primary and secondary bile acid and fatty acid biosynthesis

pathways in patients with improved MRI parameters, but future integrative analysis may provide additional insight to elucidate longitudinal changes in microbial function that tracks with disease regression or progression.

Our study has some limitations. First, although we performed prospective analyses in a well-characterized cohort of adults with biopsy-proven NASH, our sample size is modest but comparable to recent longitudinal gut microbiome studies in other disease states.[36,37] We utilized a 16 S-based sequencing approach, which provides accurate identification of bacterial communities but lacks the ability to decipher strain-level differences over time and to characterize the gut mycobiome and virome. Although all study participants were enrolled in a randomized clinical trial and advised regarding dietary intake, we cannot exclude variations in certain factors such as dietary intake and physical activity during the study period which could lead to short-term changes in the gut microbiota.[38] Neither selonsertib or simtuzumab have been shown to result in significant change in BMI or insulin resistance (measured by homeostatic model assessment for insulin resistance (HOMA-IR) and hemoglobin A1c%) in either phase 2 or 3 studies.[7,17,39] Although there were inconsistent bacterial taxonomic shifts across SEL treatment arms, indicating no clear association between treatment exposure and microbial shifts, we did find a dosage-dependent shift trend ($p = 0.07$) toward a healthy reference gut microbiome in the SEL treatment arm and cannot completely exclude treatment exposure contributing to microbial shifts. It is worth noting that the healthy microbiome reference was derived from healthy adults participating in the Human Microbiome Project, and we have limited data on this population other than age and BMI. Further studies are needed to identify predictors of longitudinal changes in LSM with MRE. Finally, correlation between microbial shifts and longitudinal clinical improvement does not imply that these are causal relationships but does provide insight in deciphering specific species which may be relevant in the pathogenesis of NAFLD and subsequently examined in animal models.

In summary, our study is novel in that we have integrated microbiome analyses into a multicenter clinical trial, permitting microbial profiling in adults with change in disease severity, yielding a number of observations that can be validated in future prospective studies. Knowledge of the impact of NASH therapeutics on the gut microbiota, and how this correlates with therapeutic response, is essential to gain new mechanistic insight into host-microbiota crosstalk in NAFLD.

Author contribution(s)

Suzanne R. Sharpton: Conceptualization; Investigation; Methodology; Writing – original draft; Writing – review & editing.

Ondrej Podlaha: Data curation; Formal analysis; Investigation; Methodology; Writing – review & editing.

Jen-Chieh Chuang: Formal analysis; Investigation; Project administration; Writing – review & editing.

Yevgeniy Gindin: Formal analysis; Writing – review & editing.

Robert P. Myers: Conceptualization; Data curation; Methodology; Resources; Writing – review & editing.

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Conflict of interest statement

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Supplemental material

Supplemental material for this article is available online.

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