ORIGINAL ARTICLE

Dynamics of hepatic steatosis resolution and changes in gut microbiome with weight loss in nonalcoholic fatty liver disease

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Abstract

Background: Weight loss is recommended as the primary treatment for nonalcoholic fatty liver disease (NAFLD). However, the magnitude and velocity of hepatic steatosis resolution with weight loss is unclear, making it difficult to counsel patients seeking weight loss for treatment of NAFLD. The aim of this study was to determine the rate of hepatic steatosis improvement and stool microbiome changes associated with rapid diet-induced weight loss in NAFLD.

Methods: Fourteen NAFLD patients (mean $\pm$ standard deviation, body mass index [BMI] 36.4 ± 4 kg/m^2) enrolled in a 12-week meal replacement program underwent frequent measurement of Fibroscan-controlled attenuation parameter (CAP). Magnetic resonance imaging (MRI-Dixon method) for hepatic fat quantitation and stool microbiome analysis (16S rRNA gene sequencing) were completed in 11 subjects at baseline and Week 12.

Results: At Week 12, mean (95% confidence interval) weight loss was -13.4 (-15.2, -11.5)% and CAP score -26.6 (-35.6, -17.6)% (both $Ps < 0.001$). CAP scores changed at a rate of -4.9 dB/m/kg (-30.1 dB/m per unit BMI) in Weeks 1–4 and -0.6 dB/m/kg (-2.4 dB/m per unit BMI) in Weeks 8–12. MRI-determined hepatic fat fraction decreased by -74.1% ($p < 0.001$) at a rate of -0.51%/kg (-3.19% per unit BMI), with complete steatosis resolution in 90% patients. BMI change was associated with decreased stool microbial diversity (coefficient $= 0.17$; Shannon Index), increased abundance of *Prevotella_9* (Bacteroidetes; coefficient $= 0.96$) and decreased abundance of *Phascolarctobacterium* (Firmicutes; coefficient $= -0.42$) (both $Ps < 0.05$).

Conclusions: Diet-induced intensive weight loss is associated with rapid improvement and complete resolution of hepatic steatosis and decreased stool microbial diversity. These findings highlight the dynamic nature of hepatic fat and may help clinicians to develop evidence-based treatment goals for patients with NAFLD and

obesity who undertake weight loss interventions. Further research is warranted to understand the effects of intensive weight loss and gut microbiome changes on long-term NAFLD resolution.

KEYWORDS
liver disease, microbiome, obesity, weight loss

1 | INTRODUCTION

Weight loss through lifestyle modification is currently considered the most effective treatment for nonalcoholic fatty liver disease (NAFLD). Reduction in body weight by 5% is associated with 25% steatosis reduction, >7%–9% with improvement in nonalcoholic steatohepatitis (NASH), and >10% with regression of liver fibrosis.[1,2] Twelve months of intensive lifestyle intervention in patients with type 2 diabetes mellitus (DM) and 6 weeks of very low-calorie diet in subjects with obesity was shown to reduce magnetic resonance spectroscopy-determined hepatic fat.[3,4] Sustained weight loss through weight loss surgery leads to improvement in NAFLD but access and healthcare inequities have limited its widespread use.[5–7]

Despite the effectiveness of weight loss for management of NAFLD, achieving and maintaining clinically significant weight loss is challenging for most patients due to a variety of environmental and individual factors, including an obesogenic global food environment, rapid drop-off in dietary adherence to most common dietary interventions, genetic and epigenetic predisposition to developing obesity, adaptive thermogenesis, and alterations in gut bacteria.[8–12] Thus, given the promise and challenges of weight loss as a therapeutic strategy for NAFLD, it is important to understand the dynamic and complex physiologic relationship between obesity and NAFLD to improve effectiveness of weight loss interventions.

In this study, we measured the rate of improvement in hepatic steatosis, associated improvement in metabolic function, and pre- and post-treatment gut microbiome alterations in NAFLD patients undertaking a hypocaloric meal replacement weight management program. The primary purpose of this study was to determine the rate and magnitude of change in hepatic steatosis with diet-induced weight loss and whether it is possible to achieve complete resolution of hepatic steatosis in NAFLD patients through diet-induced weight loss alone.

2 | METHODS

2.1 | Patient screening and enrollment

Patients with a prior diagnosis of NAFLD and obesity (body mass index, [BMI] $\geq$ 30 kg/m^2) were recruited in this prospective, observational, proof-of-concept study from the UPMC Fatty Liver Clinic. All enrolled subjects had previously agreed to participate in a clinically indicated 12-week low-calorie diet meal replacement program (Optifast®) for weight loss. Prior to enrollment, all subjects had tested negative for other etiologies of chronic liver disease, including hepatitis B and C, alcohol use disorder, autoimmune hepatitis, Wilson's disease, hereditary hemochromatosis, and Alpha-1 antitrypsin deficiency. NAFLD was diagnosed based on abdominal imaging showing hepatic steatosis and negative workup for other etiologies of chronic liver disease. None of the patients had prior liver biopsy due to noninvasive assessment of liver fibrosis by Fibroscan®-determined vibration controlled transient elastography (VCTE) suggesting low risk of advanced liver fibrosis.[13] One patient had high VCTE liver stiffness measurement (LSM) at baseline but low Fibrosis-4 (FIB-4) and NAFLD Fibrosis scores, which are noninvasive tests with high negative predictive value for advanced liver fibrosis.[14,15] Seventeen patients signed consent, 16 started the program, two dropped out after 2 weeks, and 14 completed the weight loss program.

2.2 | Dietary intervention

The dietary intervention for weight loss consisted of the Optifast meal replacement program, a validated clinician-supervized weight loss intervention in which normal diet was substituted for the pre-scribed, nutritionally complete meal replacement program.[16] Patients were recommended approximately 1100 Calories per day, including five servings (960 Calories) of the Optifast product supplemented by two cups of nonstarchy vegetables per day for 12 weeks. Participants then transitioned to a regular diet over the subsequent 12 weeks via gradual reduction of the meal replacement product by one Optifast serving every 2 weeks and increase in regular food based on the Mediterranean-style diet.[17]

2.3 | Clinical phenotyping and biochemical measurements

We recorded baseline clinical characteristics, including age, gender, race/ethnicity, height, weight, waist circumference, BMI, and presence or absence of DM. We also recorded baseline and end-of-treatment alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, albumin, fasting glucose, fasting lipid panel, and thyroid stimulating hormone (TSH) levels. Morphometric and biochemistry changes were also recorded every 4 weeks for 12 weeks.

2.4 | Determining the dynamics of hepatic steatosis resolution

Enrolled subjects returned for follow-up visits every week for the first four weeks, and then every two weeks until 24 weeks or end of their participation. VCTE for LSM (in kilopascals [kPa]) and controlled attenuation parameter (CAP) as semiquantitative determination of hepatic steatosis (in decibels per meter [dB/m]) were performed at baseline and every follow-up visit by a single trained operator using a FibroScan® 502 Touch system (Echosens), with the choice of probe guided by the system software. VCTE measurements were considered valid if at least 10 valid measurements and interquartile range over median of <30% were obtained.[18]

2.5 | Hepatic fat quantitation by magnetic resonance imaging

Eleven subjects also completed MRI-determined hepatic fat quantitation at baseline and 12 weeks. Three subjects could not complete the MRI scan due to unanticipated reasons (two with severe claustrophobia and one with body habitus incompatible with the scanner bore). The baseline and end-of-treatment MRI and VCTE/CAP measurements were performed sequentially on the same day after a minimal of 4 h fasting. A 3.0T MR system was used. The full volume of the liver was imaged using two separate breath holds of 15 s each. A region of interest was marked in three areas of the liver for calculation of the fat fraction. The fraction of hepatic fat (fat/[fat + water]) was measured using a three-point Dixon method.[19] The Dixon three-point methods uses three separate echo times (ΔTE = 2.28, 3.42, 4.56 ms), such that the 1.3 ppm lipid resonance is acquired in and out of phase with the water resonance, enabling corrections for B0 inhomogeneity. Images were acquired using TR = 5.4 ms with a flip angle of 100, slice thickness = 5 mm, FOV = 450 × 450 mm, 480 × 480 matrix size for 0.94 × 0.94 mm in plane resolution.

2.6 | Segmentation analysis

A single axial image (from in-phase gradient recall echo T1-weighted sequence) through L2 vertebral body was used for segmentation of visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT), and paraspinal skeletal muscles. Images were analyzed by SliceOmatic software (version 4.3; Tomovision) and segmentation was performed using previously described manual thresholding technique.[20] Skeletal muscle and fat areas were expressed in cm^2. Changes in the three compartments were measured by calculating the pre- and post-weight-loss VAT/SAT and VAT/muscle ratios.

2.7 | Stool microbiome analysis

DNA extraction from stool samples collected at baseline and week 12 was performed using the Qiagen DNeasy Powersoil Kit (Qiagen Cat#12888) and processed per manufacturer's protocol. Reagent blanks were included as negative controls and cells from a microbial community of known composition (ZymoBiomics Microbial Community Standards; Zymo Research) as a positive control. Extracted gDNA was amplified using Q5 HS High-Fidelity polymerase (New England BioLabs). Approximately 5 ng DNA of each sample were amplified in 25 μl reactions. Cycle conditions were 98°C for 30 s, then 30 cycles of 98°C for 10 s, 57°C for 30 s, and 72°C for 30 s, with a final extension step of 72°C for 2 min. Amplicons were purified with AMPure XP beads (Beckman Coulter) at a 0.8:1 ratio (beads:DNA) to remove primer-dimers. Eluted DNA was quantitated on a Qubit fluorimeter (Life Technologies). Sample pooling was performed on ice by combining 40 ng of each purified band. For negative controls and poorly performing samples, 20 μl of each sample was used. The sample pool was purified with the MinElute PCR purification kit (Qiagen). The final sample pool underwent two more purifications—AMPure XP beads to 0.8:1 to remove primer dimers and a final cleanup in Purelink PCR Purification Kit (Life Technologies Cat #K310001). The purified pool was quantitated in triplicate on the Qubit fluorimeter prior to sequencing.

The sequencing pool was prepared as per Illumina's recommendations, with an added incubation at 95°C for 2 min immediately following the initial dilution to 20 pM. The pool was then diluted to a final concentration of 7 pM + 15% PhiX control. Sequencing was done on an Illumina MiSeq 500-cycle V2 Nano kit (Illumina, Inc).

2.8 | Statistical analysis

The mean values of all measurements measured at baseline (week 0) and different time points during intervention (week 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 30) were calculated. The percent change of a given measurement was calculated by ([late measurement−early measurement]/early measurement) × 100% and was tested using one sample t-test. Statistical analyses were carried out using SAS software version 9.4 (SAS Institute). All P-values reported are two-sided, and a threshold level of $p < 0.05$ was considered to be statistically significant.

For stool microbiome analysis, sequences from the Illumina MiSeq were deconvolved and then processed through the Center for Medicine and the Microbiome's (CMM) in-house sequence quality control pipeline, which includes dust low complexity filtering, quality value trimming, and trimming of primers used for 16S rRNA gene amplification, and minimum read length filtering. Forward and reverse reads were merged into contigs then processed through the CMM's Mothur-based 16S clustering and sequence annotation pipeline.[21] Sequence taxonomic classifications were performed with

the Ribosomal Database Project's (RDP) Naive Bayesian Classifier[22] with the Silva reference database.[23]

Microbiota profiles were statistically quantified and analyzed using three distinct methods at the genus taxonomic level: Distance-based methods (intersample difference) utilized the Manhattan distance metric, distribution-based methods (e.g., diversity) utilized the Tail statistic[24] and Shannon index, and abundance-based methods utilized the additive-log ratio (alr) transformation.[25] Sample time points (baseline and twelve weeks post dietary intervention) from the same subject were paired and linear models were fit with the paired differences as the response (paired analyses). Variables included in the model were age, gender, BMI, systolic blood pressure, LSM (kPA), total bilirubin, and albumin at baseline. All microbiota-associated analyses were performed with custom R scripts.[26]

3 | RESULTS

3.1 | Baseline characteristics and changes at 12 weeks after weight loss intervention

The baseline characteristics of the study cohort are show in Table 1. At baseline, the study participants as a whole had Class II obesity with serum ALT level just above normal. Other AST and ALP and liver synthetic function were normal. After the 12-week dietary intervention, mean body weight was decreased by 13.4% ($p < 0.001$) and the BMI category decreased to Class I obesity with normalization of ALT.

As per the Optifast program guidelines and liver enzyme elevation with rapid weight loss reported in prior research,[27] we measured liver enzymes every two weeks for the first four week and monthly thereafter until Week 12. We did not observe any clinically significant increase in biomarkers of liver injury at any time point. There was a mild, clinically nonsignificant increase in total bilirubin ($P = 0.036$) after weight loss without associated change in albumin to suggest deterioration in hepatic synthetic function.

At baseline, mean fasting glucose and HgbA1c were in the pre-diabetes range and lipid panel was characteristic of NAFLD with elevated triglyceride and low HDL. At Week 12, there was significant improvement in metabolic parameters, including fasting glucose (-9.8%, $P = 0.039$), HgbA1c (-7.2%, $P = 0.022$), total cholesterol (-15.2%, $P = 0.001$), triglyceride (-26.4%, $P = 0.012$), and LDL-C (-13.2%, $P = 0.035$). There was no change in HDL-C or TSH.

Among 14 individual patients, nine had ALT ≥ 35 U/L (upper limit of normal [ULN]) at baseline; 8 of them achieved normal ALT by Week 12. All three patients with baseline AST ≥ 35 U/L ULN had normalization of AST after weight loss, whereas five out of nine patients with elevated fasting glucose at baseline achieved normal fasting glucose levels at Week 12. Total cholesterol (≥ 200 mg/dL), triglyceride (≥ 150 mg/dL), and LDL-cholesterol (≥ 130 mg/dL) levels were elevated above ULN at baseline in 7, 9, and 6 patients, respectively; 3, 6, and 3 patients reduced their lipid levels below ULN, respectively, at Week 12.

3.2 | Dynamics of hepatic steatosis resolution with weight loss

Mean CAP score was in the severe (S3) steatosis category but the mean LSM was <7 kPa (none or minimal liver fibrosis). At Week 12, means CAP score significantly decreased from 341 dB/m to 251 dB/m, a -26.6% change ($p < 0.001$). There was no significant change in mean LSM values after weight loss at Week 12.

Hepatic steatosis, as measured by mean CAP scores, improved rapidly in the first four weeks of the dietary intervention, followed by a slower rate of improvement until week 12 (Figure 1). The rate of change in CAP scores was -4.9 dB/m/kg weight loss (or -30.1 dB/m per unit BMI decline) in the first 4 weeks followed by -0.6 dB/m/kg weight loss (or -2.4 dB/m per BMI decline) in Weeks 8–12.

At Week 12, the mean BMI was -13.5% and associated with -26.6% change in CAP score. Despite continued modest weight loss from Weeks 12–24 during the dietary transition phase, there was nonsignificant increase in CAP score. There was no significant change in LSM values at either the 12- or 24-week time points.

Hepatic fat quantitation measured by MRI was available for 11 patients at baseline and Week 12 (Table 2). The mean ($\pm$SD) hepatic fat fraction at baseline was 18.8 (±7.5)% (Grade 2 hepatic steatosis), and 4.3 (±3.6) % at Week 12 (normal). Ten (90%) patients had complete resolution of hepatic steatosis, defined as <6% fat at Week 12. When measured by MRI-Dixon, rate of change in hepatic fat fraction was 0.51%/kg (or 3.19% per unit BMI decrease) over 12 weeks. On a qualitative review of the MRI scans the improvement in hepatic fat was uniformly distributed over the entire liver and quantitatively similar values across the three voxels selected from different parts of the liver section (Figure 2A).

We next evaluated changes in VAT, SAT, and muscle at the L2 vertebral level at baseline and Week 12. The mean change in VAT and SAT were -23.5%, and -20.7%, respectively (both $p < 0.001$), with a nonsignificant -4.4%, change in muscle area (Table 2). Weight loss did not change the VAT/SAT ratio but there was a significant reduction in the VAT/muscle ratio at Week 12.

We determined correlation between MRI-determined hepatic fat fraction and VAT, SAT, and muscle area. At baseline, hepatic fat was significantly correlated with SAT but not with VAT or muscle measurements (Table 3).

Individual morphometric differences in hepatic steatosis at baseline and response to weight loss intervention.

We next evaluated individual differences in baseline BMI and hepatic fat content in response to weight loss. There was a wide range in BMI and hepatic fat content at baseline. Despite these wide differences, 10 of the 11 patients had complete resolution of hepatic steatosis (hepatic fat fraction <6% by MRI) at Week 12 (Figures 2B and 2C).

In all 14 patients with available data, there was a significant decrease in CAP score during Weeks 0–12, after which the values remained below threshold values for hepatic steatosis in all but 2 patients. Using the manufacturer's recommended cutoff of 248 dB/m

TABLE 1 Baseline clinical characteristics and changes at 12 weeks after weight loss intervention initiation ($n = 14$)

	Baseline mean (SD)	Week 12 mean (SD)	% Change from baseline to week 12 (95% CI)	p-value
Weight (kg)	103.87 (18.05)	90.26 (18.09)	−13.4 (−15.2, −11.5)	<0.001
BMI (kg/m^2)	36.4 (4.0)	31.6 (4.0)	−13.5 (−15.2, −11.7)	<0.001
Waist circumference (cm)	117.8 (11.9)	103.6 (8.1)	−10.6 (−12.3–9.0)	<0.001
MAP (mmHg)	101.4 (7.6)	96.2 (7.7)	−4.5 (−10.7, 1.6)	0.136
ALT (U/L)	41.1 (19.4)	23.4 (11.2)	−35.6 (−52.8, −18.5)	0.001
AST (U/L)	26.7 (11.5)	20.6 (5.7)	−14.1 (−32.5, 4.3)	0.123
ALP (U/L)	73.4 (20.4)	68.6 (18.8)	−5.6 (−13.3, 2.1)	0.142
Tbili (mg/dL)	0.56 (0.23)	0.64 (0.25)	21.9 (1.7, 42.2)	0.036
Albumin (g/dL)	4.41 (0.19)	4.43 (0.15)	0.5 (−2.5, 3.5)	0.735
Glucose (mg/dL)	108.6 (30.1)	94.7 (12.8)	−9.8 (−19.0, −0.6)	0.039
TC (mg/dL)	186.6 (50.2)	157.1 (46.7)	−15.2 (−23.2, −7.3)	0.001
TG (mg/dL)	178.0 (81.2)	113.5 (50.1)	−26.4 (−46.0, −6.8)	0.012
LDL-C (mg/dL)	110.6 (44.2)	93.6 (40.9)	−13.2 (−25.3, −1.1)	0.035
HDL-C (mg/dL)	42.8 (8.2)	40.4 (7.3)	−3.7 (−15.4, 8.0)	0.502
HgbA1c (%)	6.1 (0.9)	5.5 (0.4)	−7.2 (−13.1, −1.3)	0.022
TSH (mIU/L)	2.6 (1.5)	2.3 (1.5)	−16.1 (−41.8, 9.5)	0.188
CAP (dB/m)	341.0 (33.0)	251 (64)	−26.6 (−35.6, −17.6)	<0.001
LSM (kPa)	6.5 (3.7)	5.3 (2.2)	−7.8 (−26.0, 10.5)	0.375

Abbreviations: ALP, alkaline phosphatase; ALT, aspartate aminotransferase; AST, alanine aminotransferase; BMI, body mass index; CAP, controlled attenuation parameter; HDL, high density lipoprotein cholesterol; HgbA1c, glycosylated hemoglobin; LDL, low density lipoprotein cholesterol; LSM, liver stiffness measurement; MAP, mean arterial pressure; Tbili, total bilirubin; TC, total cholesterol; TG, triglyceride; TSH, thyroid stimulating hormone.
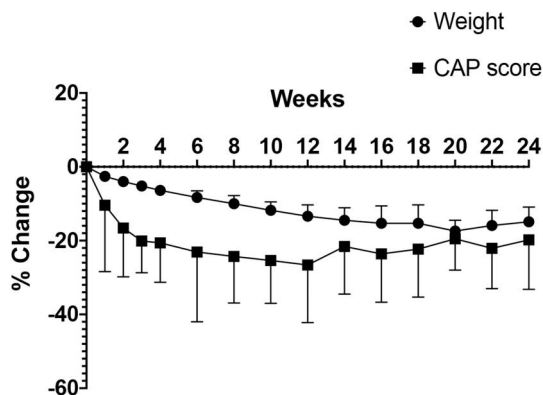

FIGURE 1 Changes in weight and controlled attenuation parameter measurements from baseline over the course of the weight loss intervention. Graph shows mean (standard deviation) percentage change measured biweekly over the course of the study

for steatosis Grade 0 versus Grades 1–3, and 7 of these patients did not achieve the threshold for resolution of steatosis at Week 12. However, when using the previously reported cutoff of 304 dB/m in a Western population, only one patient had residual hepatic steatosis at Week 12.[28] In this cohort with low LSM values due to absence of significant liver fibrosis, LSM values remained essentially unchanged throughout the intervention (Figures 2D and 2E).

3.3 | Gut microbiome changes after weight loss intervention

The changes in the gut microbiome after weight loss intervention are shown in Figure 3. Taxonomic profiles based on 16S rRNA gene sequences between the pre- and post-intervention timepoints were dominated in abundance by members of the *Bacteroidetes* phylum, *Bacteroides* and *Prevotella_9* and members of the Firmicutes phylum including *Faecalibacterium*, *Lachnospiraceae_unclassified*, and *Lachnoclostridium*. Abundance-based paired analyses, comparing Week 0 and Week 12 samples from the same subject, revealed no significant differences ($p < 0.05$) in abundant taxa, when controlling for key clinical variables, although on average, microbiota composition was more variable at baseline relative to post-intervention by intersample distance-based metrics. Paired analyses identified a significant increase in abundance of *Prevotella_9* (Bacteroidetes) with decrease in BMI (coefficient = 0.96, $p < 0.05$). In contrast, *Phascolarctobacterium* (Firmicutes), identified a significant decrease in abundance with decrease in BMI (coefficient = −0.42, $p < 0.05$).

Prevotella_9 abundance was also negatively associated with age (coefficient = −0.16, $p < 0.05$) and positively associated with higher levels of total bilirubin (coefficient = 0.04, $p < 0.05$) and albumin (coefficient = 0.34, $p < 0.05$). *Phascolarctobacterium* abundance was positively associated with age (coefficient = 0.07, $p < 0.05$) increase in kPa of LSM (coefficient = 0.03, $p < 0.05$) and males (coefficient = 1.72,

	Baseline mean (SD)	Week 12 mean (SD)	% Change (95% CI)	p-value
Hepatic fat, %	18.8 (7.5)	4.4 (3.6)	−74.1 (−86.2, −62.1)	<0.001
VAT (cm^2)	230.2 (82.8)	173.1 (58.6)	−23.5 (−32.7, −14.3)	<0.001
SAT (cm^2)	343.2 (50.0)	289.9 (79.7)	−20.7 (−27.8, −13.5)	<0.001
Muscle (cm^2)	126.0 (18.3)	119.4 (14.9)	−4.4 (−9.9, 1.0)	0.102
VAT/SAT ratio	0.71 (0.35)	0.64 (0.30)	−2.95 (−13.85, 7.94)	0.559
VAT/muscle ratio	1.82 (0.63)	1.44 (0.44)	−19.48 (−29.90, −9.06)	0.002

TABLE 2 Changes in body fat and muscle measurements by magnetic resonance imaging from baseline to Week 12 after the initiation of weight loss intervention

Abbreviations: SD, standard deviation; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; 95% CI, 95% confidence intervals.
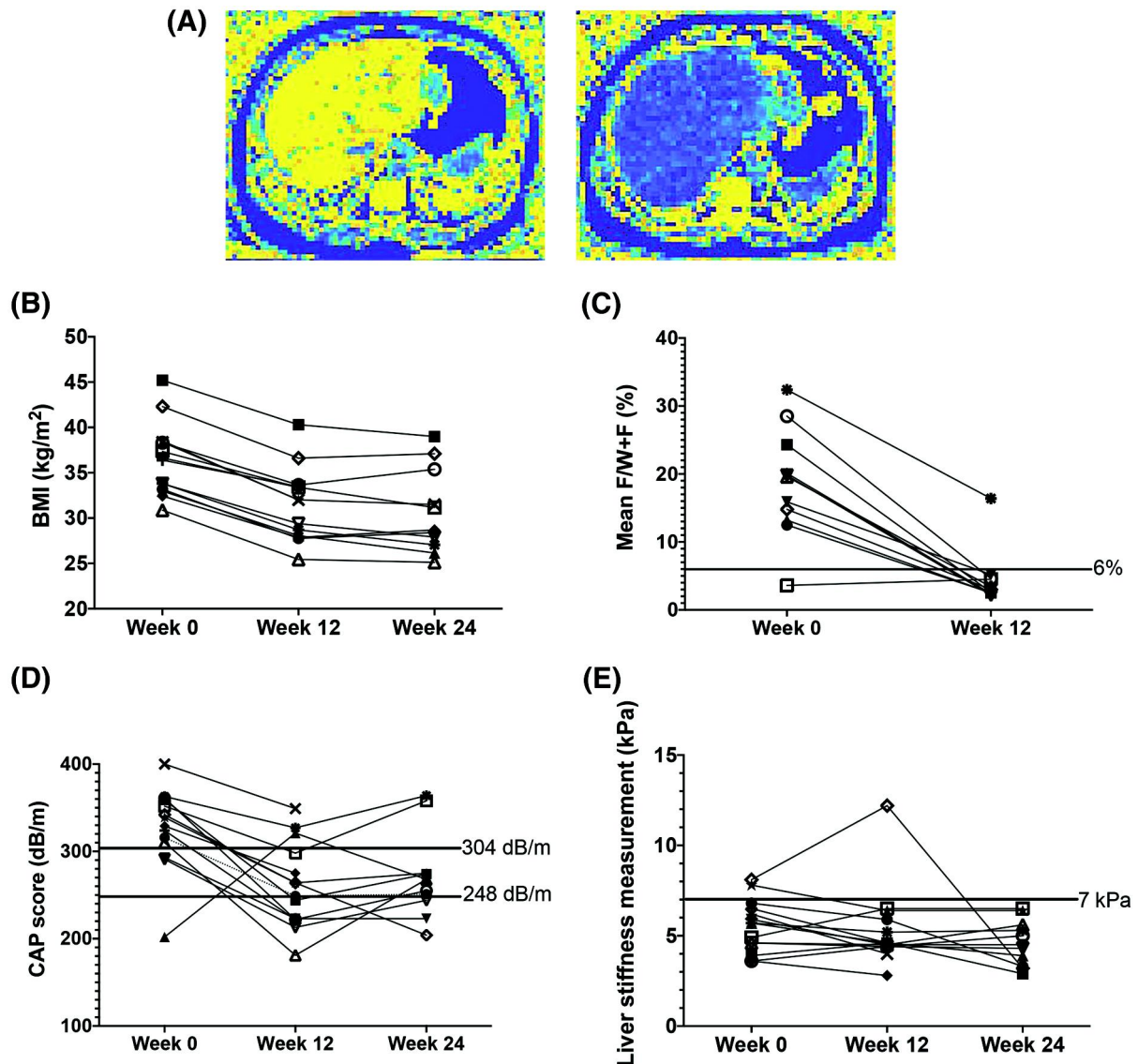

FIGURE 2 Individual differences in BMI, hepatic fat fraction, controlled attenuation parameter, and liver stiffness measurement with weight loss. (A), Example of MRI-Dixon method determined hepatic steatosis resolution with weight loss. The subject was a 51-year-old while male with a baseline BMI of 32 kg/m^2. Hepatic fat fraction decreased from 22.1% (Grade 3 steatosis; left panel) at Week 0 to 3.1% (normal liver fat fraction; right panel) at Week 12. (B), Individual BMI trajectories from 0–12 (meal replacement phase) and 12–24 weeks (transition phase). (C), MRI-Dixon method determined hepatic fat fraction at Weeks 0 and 12. The horizontal line at 6% represents the pre-specified criterion for complete steatosis resolution. (D), Hepatic steatosis via CAP measurement at Week 0, 12 and 24. The horizontal lines represent thresholds for hepatic steatosis (S0 vs. S1–3) as per the equipment manufacturer (248 dB/m) or as reported by Eddowes et al. (304 dB/m)[28] E. Liver stiffness measurement at Week 0, 12 and 24 weeks. The horizontal line represents the threshold for normal liver stiffness or absence of liver fibrosis. Abbreviations: BMI, body mass index; F/W + F, MRI-Dixon method determined hepatic fat fraction (fat/water + fat); CAP, controlled attenuation parameter; MRI, magnetic resonance imaging

TABLE 3 Spearman correlation coefficients between magnetic resonance imaging-determined hepatic fat and visceral and subcutaneous fat and muscle

	FWF	VAT	SAT	Muscle
FWF	1.00	0.288[*]	0.661[****]	0.394[**]
VAT		1.00	−0.207[*]	0.415[***]
SAT			1.00	0.248[*]

Abbreviations: FWF, fat:fat + water; VAT, visceral adipose tissue (cm^2); SAT, subcutaneous adipose tissue (cm^2).

[*] $p > 0.10$; [**] $p < 0.10$; [***] $p < 0.05$; [****] $p < 0.01$.

$p < 0.01$). *Phascolarctobacterium* abundance was negatively associated with total bilirubin (coefficient = −0.03, $p < 0.05$). *Akkermansia* (Verrucomicrobia) was also negatively associated with higher levels of total bilirubin (coefficient = −0.13, $p < 0.05$).

Paired analyses of alpha (within sample) diversity as measured by the Shannon Index identified a decrease in diversity with decreased BMI (coefficient = 0.17, $p < 0.05$). Diversity also increased with age (coefficient = 0.04) but decreased with serum albumin level (coefficient = −0.10, $p < 0.05$).

4 | DISCUSSION

Weight loss is an effective intervention for NAFLD, a condition with increasing worldwide prevalence, making it imperative to develop a clinical framework for weight management in NAFLD patients.[29] Using a noninvasive approach to frequently monitor change in hepatic steatosis during weight loss intervention, our study provides several clinical insights to guide management of coexisting NAFLD and obesity. First, hepatic steatosis is a dynamic process that improves rapidly in parallel with low-calorie diet-induced weight loss across a wide BMI and hepatic steatosis range. Second, diet-induced intensive weight loss appears to be safe and effective in low-risk NAFLD patients and our results support weight loss as an effective primary intervention for this subgroup. Third, we quantify the rate of steatosis resolution per unit weight loss for shared decision making to define therapeutic endpoints prior to any weight loss intervention for NAFLD. In addition to these clinical insights, our results demonstrate that gut microbiome changes accompany diet-induced weight loss that warrant further research to understand implications for long-term NAFLD resolution and risk of weight regain. In our study, the mean change in total body weight was −13.4% and was associated with MRI-determined hepatic steatosis resolution (defined as <6% hepatic fat fraction) in 10 out of 11 patients. These results are encouraging for clinicians and our data may motivate patients in the pre-contemplation or contemplation stage of behavior change to undertake weight loss intervention.[30]

Our study adds important clinical insights for management of patients interested in weight loss as the primary strategy for management of NAFLD. The median weight loss at 4, 8, and 12 weeks was −6.6%, −10.3% and −13.7%, respectively. However, the individual responses to the interventions were highly variable, ranging from a low of −7.9% to a high of −17.6% body weight loss. Despite achieving clinically significant weight loss, at least one patient did not have normalization of CAP score by Week 12. Furthermore, the VAT:SAT and VAT: muscle ratios did not change in the same direction for all patients, suggesting important individual differences in response to weight loss. It is also important to note that despite significant weight loss, not all patients had complete resolution of lipid and glucose abnormalities suggesting important individual variations in response to recommended levels of weight loss. Differential responses to weight loss intervention have also been reported for patients undergoing weight loss surgery and deserve further research.[31]

The microbiota is characterized by a complex arrangement of trillions of microbial cells partitioned into hundreds to thousands of species. Interest in the relationship of the gut microbiota to metabolic disorders such as NAFLD continues to grow in part due to its wide assortment of metabolic capacities, including digestion of dietary fibers, production of metabolites such as short chain fatty acids and synthesis of certain micronutrients. Here, we examined changes in microbiota composition before and after dietary intervention using 16S rRNA gene sequencing. In general, average group composition was more variable at baseline compared to post-intervention suggesting an overall influence on the gut microbiota in the context of dietary change. Compositional differences between baseline and intervention from the same individual (paired-analysis) were modest. However, this result is explained in part by our experimental design in which all individuals received the same dietary intervention, and our statistical analysis, which included controlling for the weight loss and other relevant variables. Nonetheless, *Prevotella* enrichment was associated with those individuals with the most weight loss in our study. The genus *Prevotella* are anaerobic Gram-negative bacteria of the Bacteroidetes phylum. Although the genus is large and genetically diverse, in the gut, members have been associated with diets higher in complex carbohydrates and fiber. Some studies have suggested improved glucose metabolism in association with a high-fiber diet.[32] Conversely, other studies have linked *Prevotella* with adverse physiological effects including insulin resistance.[33] Our study also indicated that the Firmicutes, *Phascolarctobacterium* was associated with weight loss, albeit by less magnitude, in our study. A previous report of a weight loss intervention associated increased abundance of *Phascolarctobacterium* with loss of at least 5% of baseline weight after three months of intervention which is at least generally consistent with our results.[34] Interestingly, our study identified a decrease in diversity with weight loss. This result is consistent with the finding that overall composition was less variable between individuals post-intervention and may further reflect responses to dietary changes. Our study is limited by our small sample size and it was not designed to specifically study the mechanism for the changes in gut microbiome. Nonetheless, the changes in microbiota composition and diversity identified here suggest that further studies are warranted of the role of the microbiome in NAFLD in the context of dietary intervention including a larger sample size and analyses of changes in microbiome function.
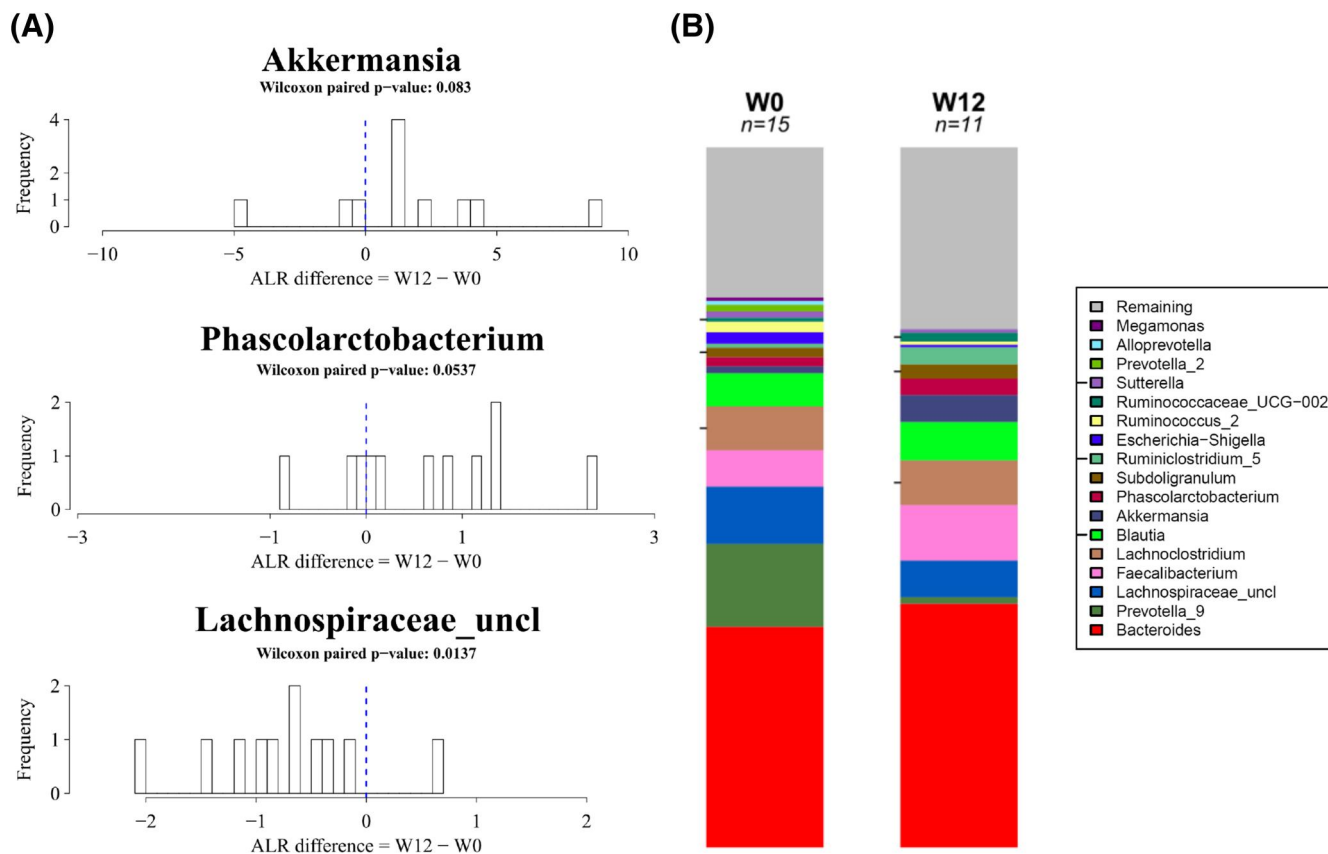
(A)

(B)

FIGURE 3 Differences in taxonomic abundance between initial and Week 12 samples. (A), These histograms represent the difference between the ALR transformed abundances of the taxon of interest between W0 and W12 samples from the same subject. The blue vertical dashed line indicates the 0 reference (no difference). A positive difference indicates an increase of taxonomic abundance over time from W0. The indicated p-values were calculated with the Wilcoxon signed-rank test, and are neither adjusted for multiple testing nor controlled for the clinical variables. (B), Stacked Bar Plots comparing initial and Week 12 taxonomic abundances. These stacked bar plots illustrate the most abundant genera as a comparison between the samples from W0 and W12. The most abundant taxa are found at the bottom of each bar. The "Remaining" contain the least abundant taxa. The three ticks on the left of the legend and bar plots correspond to the same color/taxa. additive log-ratio, (ALR)

Several limitations of our study should be noted. The enrolled NAFLD patients represented a highly motivated and low risk cohort and low-calorie meal replacement diet intervention represents a relatively difficult approach for most patients in terms of acceptability and tolerance. The reasons patients most frequently reported for declining participation included cost of the program, difficulty with frequent clinic visits for monitoring, and inability to give up favorite foods and eating patterns. Long-term weight loss maintenance remains an important clinical challenge after initial weight loss success and steatosis may recur with weight regain. CAP measurement is relatively insensitive technique for quantifying hepatic fat and may under- or over-estimate hepatic steatosis grade. We were also unable to obtain MRI data for all patients due to previously undiagnosed claustrophobia (two patients) and inability to fit in the MRI scanner (one patient).

Our study also has several strengths. This was a prospective study with frequent, pre-specified intervals of VCTE and CAP measurements allowing us to study the early dynamic changes in hepatic steatosis with clinically significant weight loss. Detailed morphometric and biochemical characterization was performed in study subjects and we correlated CAP results with MRI scans, the gold standard for measuring hepatic fat content. The study provides also provides proof-of-principal that in patients with NAFLD and obesity, early and complete resolution of hepatic steatosis is possible through dietary weight loss intervention.

In summary, we show that a 12-week low calorie diet meal replacement program achieved complete resolution of hepatic steatosis in nearly all subjects and was associated with distinctive gut microbiome changes. Further research is warranted to study the impact of rapid diet-induced weight loss and associated gut microbiome changes on long-term NAFLD resolution.

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