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Low fat but not soy protein isolate was an effective intervention to reduce nonalcoholic fatty liver disease progression in C57BL/6J mice: monitored by a novel quantitative ultrasound (QUS) method

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ABSTRACT

Untreated nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) lead to irreversible liver damage. We hypothesized that a low-fat diet (LFD) or a high-fat diet (HFD) with soy protein isolate (SPI) would be an effective intervention to halt or reverse NAFLD progression. To test these hypotheses, we conducted 2 studies. In the first study, we fed an HFD to 7-week-old C57BL/6J mice to induce NAFLD compared to an LFD (control). Hepatic steatosis was monitored by quantitative ultrasound (QUS) scans (in vivo and ex vivo). Animals were euthanized after 0, 2, 4, and 6 weeks of feeding. In the second study, 7-week-old mice were randomized onto an LFD or HFD with SPI intervention after 4 weeks of feeding HFD. Animals from each group were scanned with QUS and euthanized after 4, 9, and 12 weeks of feeding. Animals fed the HFD developed NAFLD (100%) and NASH (80%) characterized by increased liver weight, lipid accumulation, and histological scores for inflammation by 4 weeks in the first study. In the second study, the LFD ameliorated this NAFLD phenotype after 5 weeks of feeding; however, the SPI intervention failed to significantly attenuate NAFLD. QUS parameters were significantly increased with the HFDs ($P < .05$) and steatosis grade ($P < .05$) and were positively correlated with hepatic lipid concentrations. In conclusion, dietary modification may be effective at reversing NAFLD and NASH at early stages. Furthermore, QUS may become a valuable tool to track hepatic steatosis. Additional studies are needed to further evaluate the effectiveness of these interventions.

Abbreviations: ALT, alanine aminotransferase; ANOVA, analysis of variance; ATH-CAS, atherogenic diet with a casein protein source; ATH-LF, animals transferred from an atherogenic diet with casein to the low-fat diet; ATH-SOY, atherogenic diet with an isolated soy protein source; ATN, attenuation coefficient; BSC, backscatter coefficient; H&E, hematoxylin and eosin; HCC, hepatocellular carcinoma; HFD, high-fat diet; IL, interleukin; LF, low-fat dietary group; LFD, low-fat diet; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; QUS, quantitative ultrasound; SEM, standard error of mean; SPI, soy protein isolate.

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disorder in the Western world, affecting 20% of the population [1,2]. NAFLD and nonalcoholic steatohepatitis (NASH) are the result of high-fat diets (HFDs) and metabolic syndrome [3]. NAFLD includes simple steatosis (excessive accumulation of triglycerides in hepatocytes), whereas NASH is distinguished by inflammatory cell infiltration and liver cell injury. Damage to the liver by severe steatosis and NASH promotes fibrosis of the liver [4]. NAFLD and NASH are largely reversible through dietary and lifestyle modification if detected early [5]. If left untreated, NASH in particular may progress to end-stage liver disease or even hepatocellular carcinoma (HCC) [6-8]. NAFLD and NASH are the most common underlying conditions leading to HCC [9] and are projected to become the primary driver of liver transplants in developed countries [10]. Currently, NAFLD tends to be an incidental discovery that occurs while assessing other comorbid conditions [11], and there are no good assessment parameters to identify which cases of NAFLD will progress and significantly damage the liver. Improved screening techniques for treatment recommendations are needed to attenuate and reverse the burden of NAFLD and to improve liver health.

Diet is one of the most modifiable factors that contribute to the progression of NAFLD. Emerging evidence indicates that soy protein isolate (SPI) may protect the liver from steatosis and further disease progression. Notably, SPI has been shown to improve liver health and lipid homeostasis by increasing nuclear receptors and transcription factors that increase triglyceride breakdown and reduce fatty acid synthesis and fatty acid transport within the liver, leading to reduced steatosis [12-16]. Additionally, SPI has antioxidative potential and is associated with reduced inflammatory mediators in the serum and decreased inflammation within the liver [17,18]. However, the efficacy of SPI to reduce progression of NASH is not well characterized. Elucidating the potential for a dietary SPI intervention to halt or reverse disease progression could have significant clinical implications.

Clinicians also commonly recommend dietary modification to reduce the symptoms associated with comorbid conditions in NAFLD patients [5,11]. For these conditions, a low-fat diet (LFD) or energy restriction is commonly recommended to improve symptoms [19,20]. LFD interventions have also been associated with decreases in hepatic steatosis and weight loss in humans [21]. Additionally, LFDs could attenuate or reverse histological lesions associated with NAFLD through activation of adiponectin and other hormones that affect fatty acid metabolism [22]. Although LFDs are frequently recommended to patients with NAFLD, few studies have investigated their efficacy as an intervention to halt NAFLD progression without energy restriction. Among these studies, LFDs have been associated with improved liver function [22,23]. Further research is needed to clarify and understand the impact of LFDs on NAFLD progression.

We hypothesized that an SPI or low-fat dietary intervention could reduce the progression of NAFLD. To investigate the impact of these interventions on the progression of NAFLD, we conducted 2 studies. The objective of the first study was to establish the histological progression of NAFLD induced by a milk-based protein (casein) HFD in C57BL/6J mice. Furthermore, we used quantitative ultrasound (QUS) imaging to monitor changes in the liver. The diet used to induce NAFLD was moderately high in saturated and monounsaturated fats (32% total energy [kcal or kJ]), cholesterol (1.25% of weight), and cholate (0.5% of weight). The objective of the second study was to test the impact of an SPI and low-fat intervention on NAFLD progression. In this study, we fed the same HFD with casein for 4 weeks and then applied interventions of an LFD and an HFD with SPI substituted for casein to evaluate their efficiency in the attenuation or reversal of NASH progression and further steatosis. A time-course approach was used to establish the associations between changes in plasma and hepatic parameters during diet-induced NAFLD progression and remission with our QUS imaging capability. Data from these studies will provide novel information regarding the potential of low-fat and SPI interventions to reduce histologically confirmed NAFLD.

2. Methods and materials

2.1. Diets

Three experimental diets were used to test the effectiveness of isolated soy protein on reducing NAFLD progression. An AIN-93G diet (TD.94045; Harlan Teklad, Madison, WI, USA) was used as the low-fat diet. Additionally, 2 atherogenic high-fat diets (32% energy (kcal or kJ) from fat, 1.25% cholesterol by weight, and 0.5% cholate by weight) were used with different protein sources (casein: TD.150495 and SPI: TD.150496; Harlan Teklad, Madison, WI, USA). Both of the atherogenic diets were balanced for total energy, protein, fat, carbohydrates, and digestible fiber. The low-fat (AIN-93G) diet contained 20% saturated fatty acids, 22% monounsaturated fatty acids, and 58% polyunsaturated fatty acids. Each of the atherogenic diets (ATH-CAS and ATH-SOY) contained 39% saturated fatty acids, 30% monounsaturated fatty acids, and 31% polyunsaturated acids. The composition of these diets is found in Table 1.

2.2. Animals and housing

All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee (protocol 16010). Seven-week-old male C57BL/6J mice (n = 110) were ordered from The Jackson Laboratory (Bar Harbor, ME, USA) and were allowed to acclimate to the facility for 1 week. At 8 weeks of age, mice were randomized into 1 of 2 study designs as described below. Mice were individually housed under controlled conditions (12-hour light/dark cycle, 22°C, 55% humidity), were weighed weekly, and were provided food and water ad libitum. Feed intake and body weights were measured weekly.

At the termination of each study, mice were humanely exsanguinated by cardiac puncture under deep anesthesia followed by cervical dislocation to ensure euthanasia. All mice were fasted for 12 hours prior to euthanasia. At euthanasia, serum was collected and the liver was harvested. Half of the left lateral lobe of the liver was immersion fixed in 10% neutral buffered formalin for processing, embedding, sectioning, and staining with hematoxylin and eosin for

Table 1 – Dietary composition of experimental diets

Ingredient (g/kg)	Low-fat (AIN-93G) [a] TD.94045	ATH-CAS [b] TD.150495	ATH-SOY [c] TD.150496
Cocoa butter	0	75	75
Cholesterol	0	12.5	12.5
Sodium cholate	0	5	5
Casein	200	200	0
SPI	0	0	200
L-Cystine	3	3	3
Corn starch	397.5	305	305
Maltodextrin	132	132	132
Sucrose	100	100	100
Cellulose	50	50	50
Soybean oil	70	70	70
Mineral mix (AIN-93G)	35	35	35
Vitamin mix (AIN-93G)	10	10	10
Choline bitartrate	2.5	2.5	2.5
TBHQ, antioxidant	0.014	0.028	0.028

[a] Contains 18.8% energy (kcal or kJ) from protein, 63.9% energy from carbohydrates, 17.2% energy from fat.
[b] Contains 17.3% energy from protein, 50.5% energy from carbohydrates, 32.2% energy from fat.
[c] Contains 17.3% energy from protein, 50.5% energy from carbohydrates, 32.2% energy from fat. TBHQ represents tert-butylhydroquinone.

histological assessment. The right cranial lateral liver lobe was removed for ex vivo QUS scanning. The remainder of each liver was frozen for further analysis.

2.3. *Study 1: evaluation of time needed to induce NAFLD with an HFD*

Eight-week-old male C57BL/6J mice (n = 40) were randomized onto an AIN-93G diet (LF, n = 20) or a high-fat atherogenic diet with a casein protein source (ATH-CAS, n = 20). Each animal remained on their respective study diet until euthanasia. We used the novel noninvasive QUS method developed by our laboratory to monitor hepatic fat content at various stages of steatosis as described in detail for both in vivo and ex vivo conditions [24]. Each animal received liver QUS scans at 0 week (in vivo) and at euthanasia (in vivo and ex vivo). At 0, 2, 4, and 6 weeks on study, animals from each group were euthanized. Fig. 1A

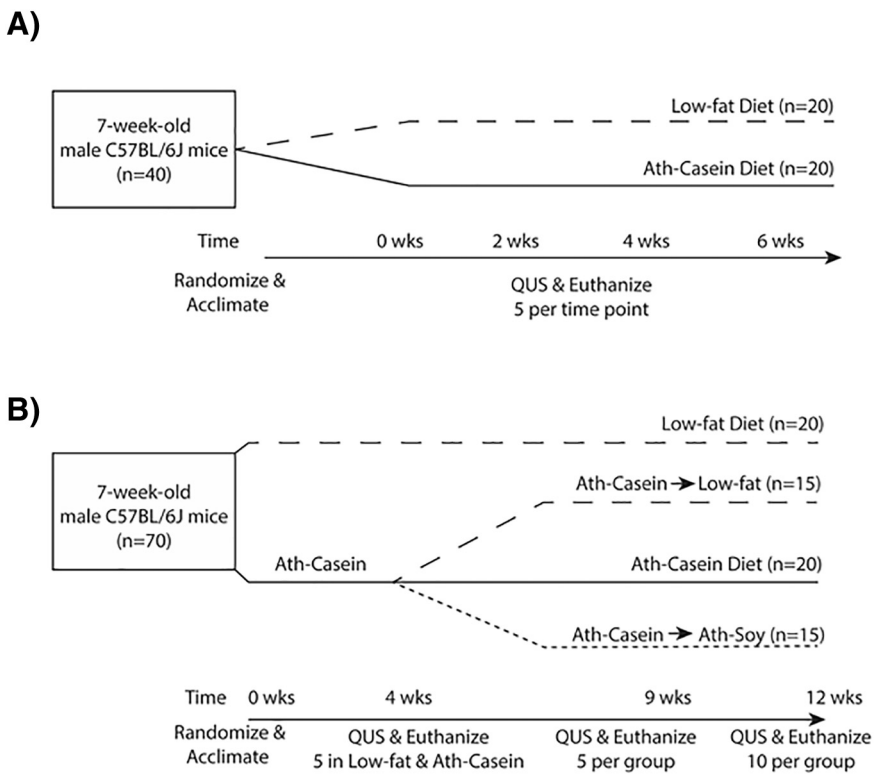

Fig. 1 – Experimental designs for studies 1 and 2. A, Design for study 1. B, Design for study 2.

shows an overview of this study scheme. This will be further referred to as study 1: the timing study.

2.4. Study 2: effect of LFD or SPI interventions on the reversal of steatosis and NASH

Eight-week-old male C57BL/6J mice (n = 70) were randomized onto an AIN-93G diet (LF, n = 20) or a high-fat atherogenic diet with a casein protein source (ATH-CAS, n = 50). After 4 weeks on the ATH-CAS diet, mice were expected to have steatosis and NASH (based on study 1). At 4 weeks, ATH-CAS animals were randomized onto 1 of 3 diets: ATH-CAS (n = 20), an atherogenic diet with an isolated soy protein source (ATH-SOY, n = 15), or LF (ATH-LF, n = 15). Each animal received liver QUS scans at the beginning of the study (0 week, in vivo), before starting the dietary intervention (4 weeks, in vivo), and at euthanasia (in vivo and ex vivo). Animals from each group were euthanized after 4 weeks (n = 5 for LF and ATH-CAS groups), 9 weeks (n = 5), and 12 weeks (n = 10) on the study. Fig. 1B shows an overview of this study scheme. This will be further referred to as study 2: the intervention study.

2.5. QUS scanning

QUS signals in tissues are based on scattering of ultrasound from cytoarchitectural features. The attenuation coefficient (ATN) is a numerical parameter that measures the energy loss in tissues that is analogous to the obscuration of tissue structures [25]. The backscatter coefficient (BSC) is a measure of ultrasound energy that is returned from tissue [25]. A higher ATN and BSC may correspond to increased lipid accumulation and other changes within the liver. For in vivo QUS imaging, the skin over the scanned area was shaved and depilated. Isoflurane (2%-5%) was used for inhalation anesthesia. Scans were obtained through the ventral body wall while in dorsal recumbency on a heated table by the 2100 platform (Vevo 2100 high-frequency imaging system; VisualSonics, Inc, Toronto, Ontario, Canada) using the MS-400 phased-array transducer (30-MHz nominal center frequency; 12- to 33-MHz bandwidth). The transducer surface, positioned perpendicular to the body, was connected to a 3-D motor. The 3-D motor was used to collect 8 parallel frames, 0.5 mm apart, in a caudal to cranial direction. Each QUS imaging frame consisted of a B-mode image along with its raw (unprocessed) radiofrequency data for each liver that was scanned. A reference phantom was scanned using the same 2100 system settings that were used to scan that liver. This reference phantom has known and spatially uniform ATN and BSC properties, which were independently calibrated. Comprehensive experimental details for the reference phantom methodology [26] that was used to acquire and process the in vivo ATN and BSC data have been published [24,27].

The right cranial lateral lobe was removed for ex vivo QUS scans. The ex vivo tissue was ultrasonically scanned with a single-element 40-MHz focused transducer (25- to 55-MHz bandwidth; *f*-number: 3; NIH High-frequency Transducer Resource Center, University of Southern California, Los Angeles, CA, USA). The transducer was connected to a UTEX UT340 pulser/receiver (UTEX Scientific Instruments Inc, Mississauga, Ontario, Canada) and moved via a computer-controlled positioning system (Daedal Parker Hannifin Corp, Irwin, PA, USA). The liver tissue was placed on a Plexiglas planar reflector within a tank filled with room-temperature degassed 0.9% saline. Comprehensive experimental details for the planar reference QUS methodology that was used to acquire and process the ex vivo ATN and BSC data have been published [24,27].

2.6. Histology

Fixed liver specimens were processed through graded alcohols, embedded in paraffin, sectioned at 3-μm thickness, mounted, and stained with hematoxylin and eosin. A board-certified veterinary pathologist (MAW), blinded to the experimental conditions, assessed the liver sections. The examined sections were assigned a score for steatosis; "balloon cell" hepatocytes (acute injury); focal lobular inflammation (indicative of previous hepatocyte death); portal inflammation (indicative of chronic inflammation); presence of large lipogranulomas (another indicator of chronic hepatic injury); degree and type of fibrosis; and diagnostic classification (0, normal; 1, mild steatosis; 2, severe steatosis; and 3, steatohepatitis). This evaluation scheme was a modification of the NASH Clinical Research Network (CRN) scoring system described by Kleiner et al [28] in the supplemental materials.

2.7. Serum and liver analyses

The serum samples were stored at –80°C until the day of the assay. Total cholesterol was measured using enzymatic colorimetric kits (Wako Chemicals, Richmond, VA, USA) according to manufacturer's instruction. Samples were plated in triplicate with human control sera (Wako Chemicals, Richmond, VA, USA) to ensure reproducibility. Serum alanine aminotransferase (ALT) was also measured by an enzymatic colorimetric kit (Biovision, Milpitas, CA, USA). Samples were evaluated in duplicate and followed the manufacturer's instruction. The serum levels of type 1 helper cell (interleukin [IL]-1β, interferon-γ, tumor necrosis factor–α), type 2 helper cell (IL-6 and IL-10), and type 17helper cell (IL-17A) cytokine profiles were measured using a Bio-Plex multiplex assay (Bio-Rad, Hercules, CA, USA). The samples were measured in duplicate, and this assay was run with accordance to the manufacturer's instruction on a Bio-Rad Luminex Cytometric Bead Analyzer plate reader. Total lipids were extracted from an average of 0.37 g of frozen liver using 1:1 chloroform-methanol and quantified by weight as previously described [29,30].

2.8. Statistical analyses

Statistical analyses were performed in SAS 9.4 (SAS Institute, Inc, Cary, NC, USA) for Windows. Repeated-measures analysis of variance (ANOVA) was used for analysis of body weight and energy intake data over time. Individual post hoc contrast statements were used to compare body weight and energy intake at each time point under each dietary condition. QUS data, tissue weights, liver fat fraction, serum ALT, serum cholesterol, and serum cytokines were compared using 1- or 2-way ANOVA with multiple-comparison adjustments by the Tukey method. Data were log transformed when assumptions

of normality were not met. Histological scores were assessed by Wilcoxon rank-sum test whenever 2 samples were present in a given time point. Otherwise, the nonparametric Kruskal-Wallis test was performed with multiple comparisons by Dunn test. A P value < .05 was considered statistically significant. All values are reported as mean ± standard error of mean (SEM).

3. Results

3.1. Study 1: evaluation of time needed to induce NAFLD with an HFD

3.1.1. The Western diet induces NAFLD and NASH

Animals on the low-fat diet generally did not develop NAFLD or NASH, as only 1 animal on the LFD was histologically diagnosed with NAFLD by the end of 6 weeks (Table 2). The atherogenic HFD (ATH-CAS) induced a NASH phenotype characterized by increased hepatic lipid deposition and increased liver weight (Fig. 2A and B). Hepatic lipid levels (a marker of steatosis) increased by ~70% with the atherogenic diet. This was reflected by an increased steatosis score (Table 2). Histological analysis also revealed significant increases in portal and lobular inflammation among animals given the atherogenic diet (Table 2). This resulted in a diagnostic classification of NASH in 80% and NAFLD in 100% of animals after 2 weeks (Table 2) and was maintained through the remainder of the study.

The ATH-CAS diet led to a significant increase in liver weight following 2 weeks of feeding and liver weights that are 40% higher than LF at each time point after baseline (P < .05, Fig. 2A). There was no difference in liver weights compared to baseline among LF animals. Similarly, the percent of liver lipids by weight increased in the ATH-CAS–fed animals but did not increase in LF animals (P < .05, Fig. 2B). By 4 weeks, there was an average of 17.9% lipid in the liver of the ATH-CAS–fed animals and 8.9% in LF animals, which remained significantly different until the end of the study.

3.1.2. QUS parameters were correlated with increased steatosis

There was no effect of diet on the in vivo QUS parameters (ATN and BSC). Although there were no significant changes in the ATN and BSC by diet (Supplemental Fig. S2A and B), the ATN was significantly correlated with the amount of lipid in the liver (Table 3). One animal had a notably high BSC (~11-fold higher than the others). After removing this animal, both of the QUS parameters were significantly correlated with the lipid in the liver (data not shown). Ex vivo QUS parameters were more strongly correlated with hepatic lipid content (P < .0001). Additionally, ex vivo ATN and BSC were significantly higher in mice fed the ATH-CAS diet (P < .05). By 4 weeks, there was a significant increase in both QUS parameters, which remained higher until the end of the study.

3.2. Study 2: effect of LFD or SPI interventions on the reversal of steatosis and NASH

3.2.1. Dietary interventions to attenuate NASH features

Similar to the first study, feeding HFD (ATH-CAS) led to the development of NASH in 60% of animals and NAFLD in 100% of animals after 4 weeks (Table 4). On average, low-fat–fed animals consumed 20% more energy (kcal or kJ) per day than ATH-CAS–fed animals (13.8 kcal/d [57.7 kJ/d] compared to 11.5 kcal/d [48.1 kJ/d], Supplemental Fig. S1C). As a result, the body weight of animals fed the low-fat diet gradually increased, whereas animals fed the Western (ATH-CAS) diet maintained a similar body weight during the experimental period (Supplemental Fig. S1D). This may have been related to the presence of cholesterol and cholate in the high-fat diets [31]. However, the atherogenic diets led to significant increases in liver weight compared to the low-fat diet (Fig. 3A). By the end of the study, the liver weight was highest in the ATH-SOY intervention group and lowest in the ATH-LF (low-fat) intervention group (Fig. 3A). Biochemical examination showed that lipids accumulated in the liver at similar proportions to study 1 at 4 weeks. ATH-CAS animals had a higher accumulation of lipids in the liver at 9 and 12 weeks

Table 2 – Histological data from study 1

	Week 0			Week 2			Week 4			Week 6		
	Low fat	ATH-CAS	P value	Low fat	ATH-CAS	P value	Low fat	ATH-CAS	P value	Low fat	ATH-CAS	P value
Diagnostic classification	0±0	0±0	NS	0±0	2.2±0.2	**<.05**	0±0	2.6±0.9	**<.01**	0.4±0.2	2.8±0.4	**<.001**
Steatosis grade	0±0	0±0	NS	0±0	1.6±0.1	**<.001**	0±0	1±0.7	**<.01**	0.2±	1±0	**<.05**
Lobular inflammation	0±0	0±0	NS	0±0	1.8±0.2	**<.05**	0.2±0.1	1.8±0.8	**<.01**	0±0	1±0.7	**<.05**
Portal inflammation	0±0	0±0	NS	0±0	1±0.1	**<.05**	0±0	1.2±0.4	**<.01**	0±0	0.6±0.5	**<.05**
Ballooning cell injury	0±0	0±0	NS	0±0	0.6±0.1	**<.05**	0±0	0.2±0.4	NS	0±0	0±0	NS

NS, not significant.
Histological data for animals in the low-fat and Ath-Casein diets (n = 5 per time point). A diagnostic classification score of 0 represents normal, 1 represents inflammation without steatosis, 2 represents steatosis, and 3 represents steatohepatitis. A steatosis grade of 0 represents <5% parenchymal involvement by steatosis; 1 represents 5%–33%, 2 represents >33%-66%, and 3 represents >66%. A ballooning liver cell injury score of 0 represents none, 1 represents mild, and 2, many cells/prominent ballooning. A portal inflammation score of 0 represents none, 1 represents mild, and 2 represents more than mild. A lobular inflammation score of 0 represents no foci per 20x field, 1 represents <2 foci per 20x field, 2 represents 2-4 foci, and 3 represents >4 foci. All data are expressed as mean ± SEM. The Wilcoxon's Rank-Sum Test was used for statistical analysis. Bolded values represent a statistically significant p-value. NS represents not significant.

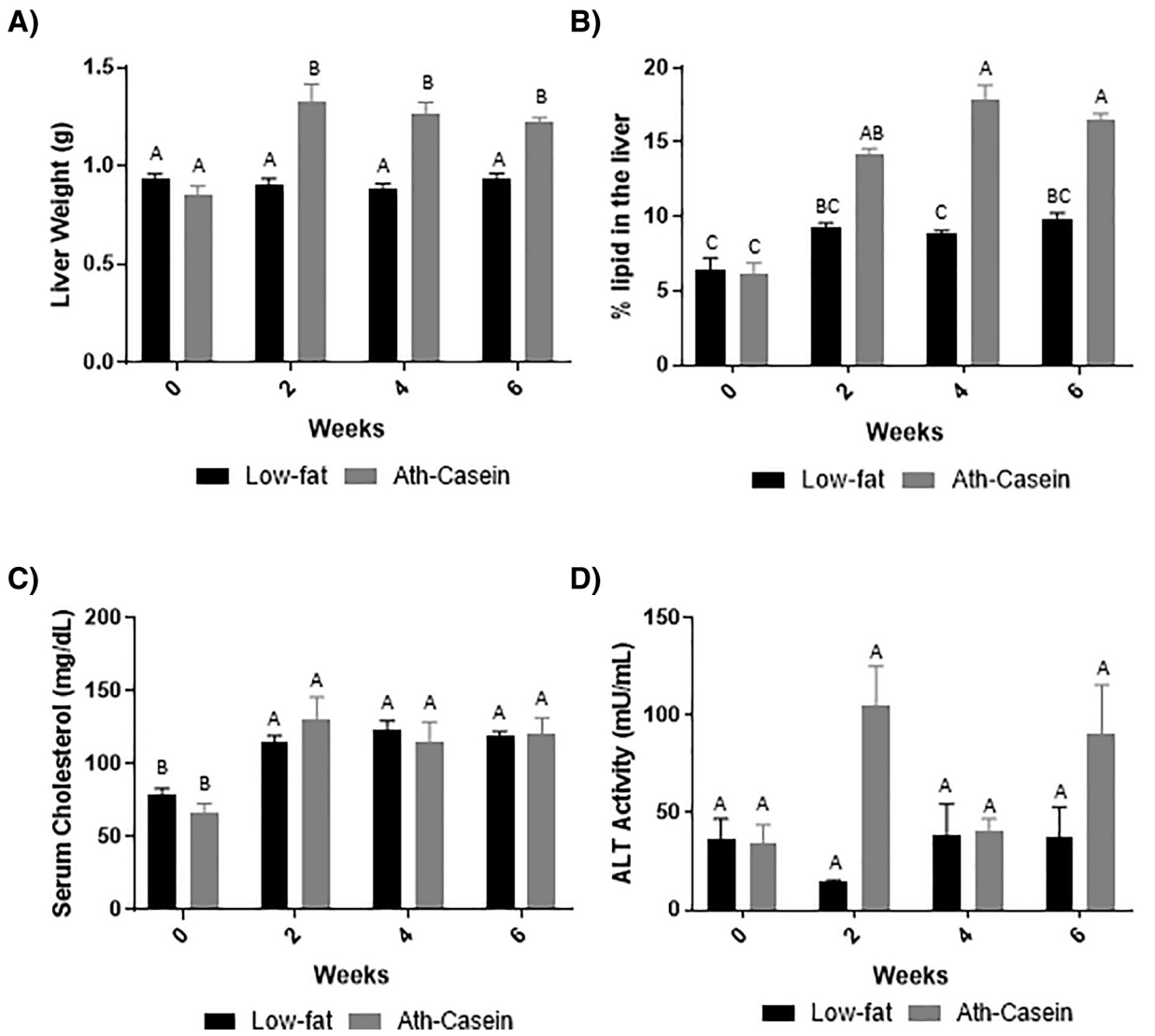

Fig. 2 – Animal characteristics from study 1. Animal characteristics from animals in the low-fat and ATH-CAS diets (n = 5 per time point). A, Liver weight, (B) lipid accumulation in the liver, (C) serum cholesterol, and (D) serum ALT. All data are expressed as mean ± SEM. Two-way ANOVA with Tukey post hoc test was used for statistical analysis. Means with different letters are significantly different from each other.

($P < .05$, Fig. 3B) compared to either LFD group (LF or ATH-LF). Interestingly, the SPI intervention group was not significantly different from any dietary treatment at 9 and 12 weeks (Fig. 3B). These observations were consistent with the histological assessment for steatosis (Table 4).

The ATH-CAS diet led to significantly higher serum cholesterol concentrations by the end of the study compared to either low-fat (LF and ATH-LF) dietary condition (Fig. 3). Serum ALT, a marker of liver injury [32], was generally higher in all high-fat dietary conditions than low-fat conditions, suggesting hepatocellular damage in addition to hepatic steatosis (Table 4). Histological analyses also revealed that the ATH-CAS diet led to significant increases in portal and lobular inflammation as early as 4 weeks in

Table 3 – Correlations between hepatic lipids and QUS

	In vivo				Ex vivo			
	ATN		BSC		ATN		BSC	
Study 1	$r = 0.43$	$P = .005$	$r = 0.17$	$P = .306$	$r = 0.76$	$P < .0001$	$r = 0.74$	$P < .0001$
Study 2	$r = 0.24$	$P = .047$	$r = 0.16$	$P = .183$	$r = 0.74$	$P < .0001$	$r = 0.14$	$P = .245$

Correlation of hepatic lipid concentrations and QUS attenuation coefficients. The coefficient of correlation of Pearson, r, and the P values are indicated.

Table 4 – Histological data for study 2

	Week 4		Week 9				Week 12			
	Low fat	ATH-CAS	Low fat	ATH-CAS→low fat	ATH-CAS	ATH-CAS→soy	Low fat	ATH-CAS→low fat	ATH-CAS	ATH-CAS→soy
Diagnostic classification	0.6±0.2^{a}	2.6±0.2^{b}	1.8±0.5^{a}	1.6±0.4^{a}	2.6±0.4^{a}	2.2±0.5^{a}	2.2±0.1^{ab}	1.3±0.4^{a}	2.9±0.1^{bc}	3.0±0^{c}
Steatosis grade	0±0^{a}	1.4±0.2^{b}	0.8±0.2^{a}	0.8±0.2^{a}	1.4±0.5^{a}	1.2±0.4^{a}	1.5±0.2^{ab}	0.6±0.2^{a}	1.4±0.2^{b}	2.1±0.3^{ab}
Lobular inflammation	0.6±0.2^{a}	1.2±0.4^{a}	0.2±0.2^{ab}	0±0^{a}	1.4±0.2^{b}	0.7±0.4^{ab}	0.4±0.2^{a}	0±0.3^{a}	1±0.3^{ab}	1.3±0.1^{b}
Portal inflammation	0±0^{a}	1.6±0.2^{b}	0.4±0.2^{ab}	0±0^{a}	1.2±0.4^{b}	0.7±0.2^{ab}	0.5±0.1^{a}	0.4±0.1^{ab}	1±0.1^{b}	0.8±0.1^{ab}
Ballooning cell injury	0.5±0.2^{a}	0.4±0.2^{a}	0.4±.02^{ab}	0±0^{a}	0.8±0.2^{b}	0±0^{a}	0.7±0.2^{a}	0.3±0.1^{a}	0.9±0.2^{a}	0.6±0.2^{a}

Histological data for animals in the Low-fat (n = 20, 5 animals at week 4, 5 animals at week 9, and 10 animals at week 12), Ath-Casein→ Low-fat (n = 15, 5 animals at week 9 and 10 animals at week 12), Ath-Casein diet (n = 20, 5 animals at week 4, 5 animals at week 9, and 10 animals at week 12), and Ath-Casein→Ath-Soy (n = 15, 5 animals at week 9 and 10 animals at week 12). A diagnostic classification score of 0 represents normal, 1 represents inflammation without steatosis, 2 represents steatosis, and 3 represents steatohepatitis. A steatosis grade of 0 represents <5% parenchymal involvement by steatosis; 1 represents 5%–33%, 2 represents >33%–66%, and 3 represents >66%. A ballooning liver cell injury score of 0 represents none, 1 represents mild, and 2, many cells/prominent ballooning. A portal inflammation score of 0 represents none, 1 represents mild, and 2 represents more than mild. A lobular inflammation score of 0 represents no foci per 20x field, 1 represents <2 foci per 20x field, 2 represents 2-4 foci, and 3 represents >4 foci. All data are expressed as mean ± SEM. Kruskal-Wallis test was performed with multiple comparisons by Dunn's test for statistical analysis. Means without the same letter in the same week are significantly different.

this study (Table 4), which are key features of developing NASH. Circulating proinflammatory cytokines were also measured but were not altered by the diet (Supplemental Fig. S3).

3.2.2. *QUS parameters were correlated with increased steatosis*

LF animals had a significant increase in their in vivo BSC at 12 weeks. Other than this time point, there was no significant effect of diet on the in vivo QUS parameters. After removing the liver (ex vivo), there was an increase in sensitivity, and ATN was significantly increased with HFD (Supplemental Fig. S4). This was further validated when stratifying animals by their steatosis score. In vivo and ex vivo ATNs significantly increased with increasing steatosis ($P < .05$, Fig. 4). The BSC also experienced some increases for the ex vivo conditions, but this was not observed in vivo. The QUS parameters were also significantly correlated with the quantity of lipid in the liver (Table 2). Specifically, ex vivo ($P < .0001$) and in vivo ($P < .05$) ATNs were positively correlated with liver lipids.

4. Discussion

NAFLD is very prevalent and is a substantial worldwide public health concern [33]. NAFLD begins as simple steatosis and can progress into NASH, cirrhosis, and HCC. In these studies, we investigated the potential for 2 dietary interventions (low fat or soy protein) to reduce or reverse the progression of steatosis and NASH. This was monitored and evaluated biochemically, histologically, and through the use of novel QUS methods. In this study, a casein-based HFD (with cholesterol and cholate) induced NAFLD in mice after 4 weeks of feeding. Longer periods of feeding led to more severe histological lesions. Histological markers of steatosis and portal and lobular inflammation all intensified with time. Similarly, hepatic lipid accumulation increased with time. In the second study, dietary modification modulated the trajectory of NAFLD. These data highlight the importance of diet and the potential that these interventions may have on NAFLD progression.

Among the possible animal models, we selected the C57BL/6J mouse as a model for our investigation of the impact of an atherogenic HFD-induced model of NAFLD. This is a well-established murine model that recapitulates many aspects of human NAFLD and NASH [31,34]. Among animal models, male C57BL/6J mice develop the most inflammation and necrosis, making it a particularly appropriate model for histological features of NASH [31]. If left untreated, NASH in particular may progress to end-stage liver disease and is one of the final reversible stages of NAFLD. This is a critical stage of NAFLD progression to intervene at before additional damage to the liver occurs. The validated assays, history of use, and histological techniques make this model a sensitive system to investigate the role of each dietary intervention on NAFLD progression. Additionally, these same factors make this model ideal to test for biological effects of NAFLD on QUS parameters.

Low-fat diets are one of the most commonly recommended treatments for NAFLD and NASH. At these stages, insults to the liver are still reversible and may be resolved through dietary changes. By the time of the designed interventions, most animals had NASH and all had NAFLD (Study 2). At 4 weeks, when animals were expected to have NASH and steatosis, 1 group was placed onto a low-fat diet. Our data suggest that an LFD intervention is able to reverse the effects of an HFD on the liver. As we hypothesized, there was no difference between the LFD intervention group and the life-long LFD group in all analyzed categories after 5 or 7

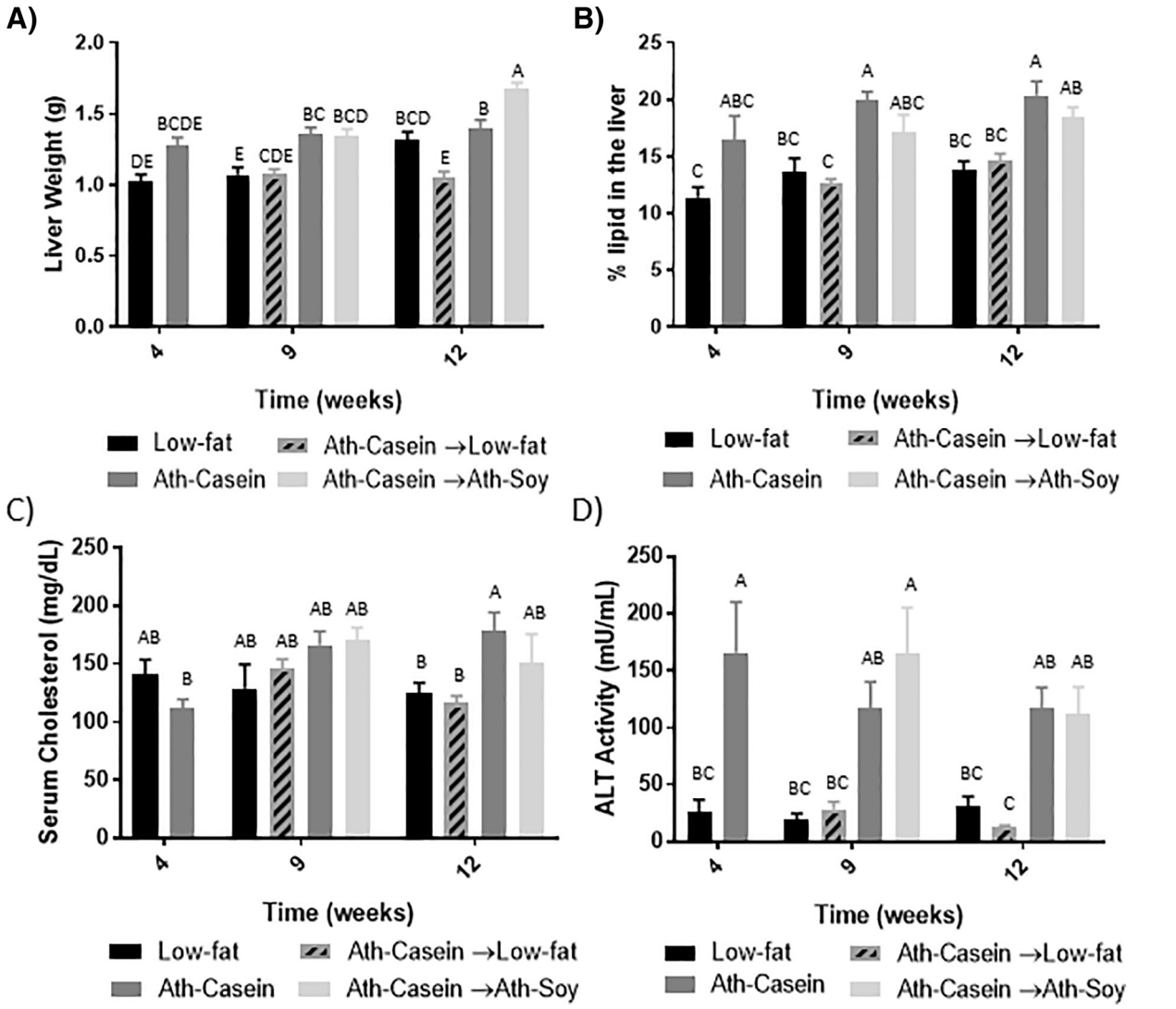

Fig. 3 – Animal characteristics from study 2. Animal characteristics from animals in the low-fat diet (n = 20, 5 animals at week 4, 5 animals at week 9, and 10 animals at week 12), ATH-CAS→low-fat diet (n = 15, 5 animals at week 9 and 10 animals at week 12), ATH-CAS diet (n = 20, 5 animals at week 4, 5 animals at week 9, and 10 animals at week 12), and ATH-CAS→ATH-SOY diet (n = 15, 5 animals at week 9 and 10 animals at week 12). A, liver weight, (B) lipid accumulation in the liver, (C) serum cholesterol, and (D) serum ALT. All data are expressed as mean ± SEM. Two-way ANOVA with Tukey post hoc test was used for statistical analysis. Means with different letters are significantly different from each other.

weeks of the intervention. Thus, this low-fat intervention may alter the lipid regulation in the liver and promote healthier function. Other studies have shown similar results prior to the onset of liver fibrosis [22]. However, LFDs were not able to overcome all of the damage to the liver after the onset of liver fibrosis [23]. These results emphasize the importance of detecting NAFLD and intervening at early stages to reduce disease burden.

In addition to a low-fat intervention, we also sought to elucidate the impact of an SPI intervention on NAFLD progression. The intervention diet was still atherogenic, but soy protein replaced the casein protein in the diet. Contrary to our hypothesis, replacing the protein source after 4 weeks of feeding failed to significantly reverse hepatic steatosis, inflammation, liver damage, or histological markers of NASH. Although no significant liver lipid reductions were observed, the soy intervention was not significantly different compared to the lifelong LFD across any biochemical marker. Although we rejected our hypothesis that SPI can reduce the progression of NAFLD, it is possible that a longer intervention length may have led to significant reductions in our markers of NAFLD. One study found that longer periods of feeding a diet containing SPI (16 weeks compared to 8 weeks) led to greater reductions in hepatic steatosis and serum ALT levels [35]. Our intervention was shorter (8 weeks) and may have not been long enough to reduce markers of NAFLD in this study. Other preclinical data suggest that SPI may be protective against NAFLD by reducing inflammation and hepatic lipid transport [12,13]. One clinical trial found that a low-fat diet with SPI had significant benefits for patients with NAFLD [14]. Other studies support its role as a prophylactic [35–37], but few have investigated its role as an intervention for NASH.

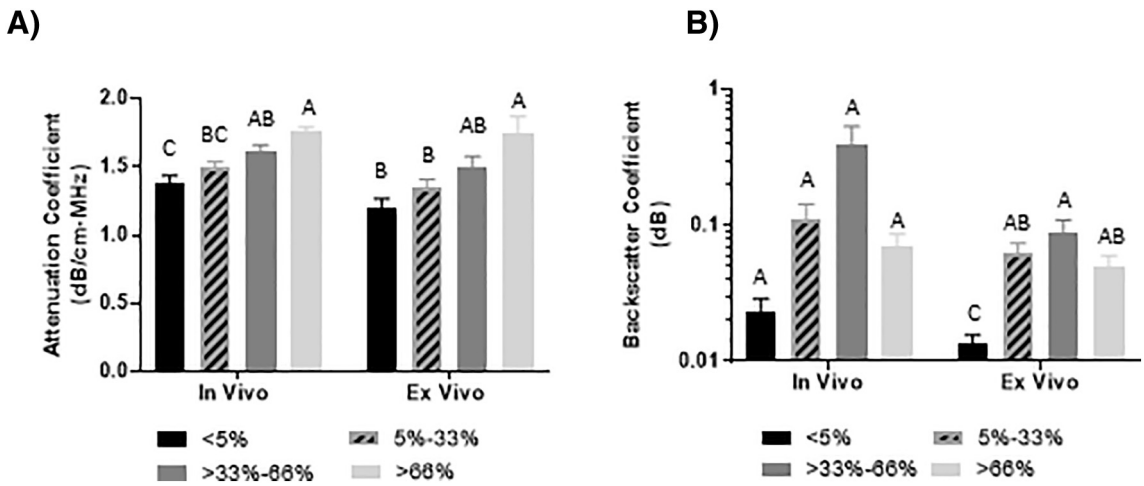

Fig. 4 – QUS parameters from study 2. Average QUS parameters across steatosis grade categories in the low-fat diet (n = 20, 5 animals at week 4, 5 animals at week 9, and 10 animals at week 12), ATH-CAS→low-fat diet (n = 15, 5 animals at week 9 and 10 animals at week 12), ATH-CAS diet (n = 20, 5 animals at week 4, 5 animals at week 9, and 10 animals at week 12), and ATH-CAS→ATH-SOY (n = 15, 5 animals at week 9 and 10 animals at week 12). A, Average ATN by steatosis grade, (B) BSC by steatosis grade. One-way ANOVA with Tukey post hoc test was used for statistical analysis of each in vivo or ex vivo condition. Means with different letters are significantly different from each other.

Future studies are needed to determine the efficacy of an SPI intervention to reduce the burden of NAFLD and NASH.

These studies provide insights into the potential impact of a low-fat or SPI intervention to reduce NAFLD progression. The impact that an intervention may have on the liver depends on the stage of NAFLD. These interventions were initiated when all animals had histologically confirmed NAFLD. Many animals also had histologically confirmed NASH. The current standard for NAFLD detection involves a liver biopsy [11], which is invasive and is not recommended for screening healthy individuals [38]. The development of a sensitive, noninvasive, and real-time method to monitor hepatic steatosis could be a significant tool with a high impact on clinical care [24,25,39–42]. Hepatic ATN and BSC measures with a reference phantom technique on clinical ultrasound machines are repeatable and reproducible across transducers and sonographers in adults with NAFLD [25,42]. QUS imaging increases the specificity of the ultrasound imaging through quantitative outcomes and can lead to improvements in diagnostic ultrasound [43]. The use of QUS may improve the noninvasive diagnosis of NAFLD.

`Lipid in the liver has been shown to affect both ultrasonic ATN and BSC [44]. We hypothesized that increased hepatic steatosis would increase the ATN and BSC from QUS. In the current studies, hepatic lipid concentrations were significantly correlated with *in vivo* ATN. Additionally, the average in vivo ATN significantly increased as steatosis score increased. Although statistically significant differences were found for mean ATN and BSC values across scores for steatosis (in vivo or ex vivo), there was still overlap within each steatosis score stratum. Other factors, such as inflammation, may also affect QUS parameters of the liver. This has been observed in other tissues [45–47]. More data are needed to validate the use of this technique to monitor early stages of NAFLD.

These studies exhibit several strengths that contribute to the literature regarding the impact of SPI and LFD interventions on NAFLD and NASH progression. The effectiveness of these interventions was monitored and evaluated biochemically, histologically, and through the use of novel QUS methods. Each dietary intervention was initiated after histologically confirming that animals had NAFLD (study 1). To our knowledge, there are currently no studies of low fat or SPI as a method to reverse the early stages of NAFLD prior to the onset of fibrosis in a mouse model. These studies enhance the existing literature by testing whether 2 different dietary interventions could reduce or reverse NAFLD prior to fibrosis.

Despite these strengths, these studies have a number of limitations that should be considered. These studies were only conducted in male C57BL/6J mice. The severity of NASH in rodents fed atherogenic diets depends on the sex of the animal. One study found that male C57BL/6J mice had a higher NAFLD activity score than female C57BL/6J mice [48]. Furthermore, this study found that exogenous testosterone provided to castrated mice led to increased NAFLD and liver damage, whereas exogenous estrogen alleviated pathological damage caused by NASH [48]. We do not expect that the low-fat intervention would have had a differential impact on different sexes of mice. SPI contains isoflavones that stimulate Wnt signaling through an estrogen receptor–dependent pathway [49], which may have a different impact on different sexes of mice. This may lead to differential results regarding the impact of the SPI intervention that were not captured in this study. Future studies are needed to investigate the potential impact that sex may have on these dietary interventions. The low sample size of this study may have also limited our ability to determine which factors contributed to the dependencies with QUS parameters.

In conclusion, a low-fat dietary intervention was sufficient to reverse NAFLD. Future studies are needed to elucidate whether a longer SPI intervention would improve markers of NAFLD. The effectiveness of these interventions may have been attributed to alterations in hepatic lipid accumulation

and inflammation. Importantly, our QUS parameters were significantly correlated with increasing hepatic lipid concentrations and histologically defined steatosis. These findings reveal the potential for a novel method of monitoring and tracking steatosis within the liver. A noninvasive and real-time method of tracking steatosis could lead to earlier diagnoses where these interventions would be particularly effective to prevent the progression of NAFLD.

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Supplemental materials

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