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NAFLD-related SNPs are linked to changes in liver fat, as measured by the CAP score, and serum lipids in response to a 3-week sugar-sweetened beverage intervention: a pilot study

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Studies show that increased consumption of sugar-sweetened beverages (SSBs) is linked to non-alcoholic fatty liver disease (NAFLD), a condition characterized by excess fat accumulation in hepatocytes. Genetic factors also influence NAFLD. We conducted a clinical trial (NCT03783195) to determine if SNPs related to NAFLD are associated with liver fat content and its changes in response to a 3-week SSB intervention in Caucasian adolescents and young adults. Fifteen participants (5 males and 10 females, mean age 25.5 ± 9 years) consumed a beverage daily for 3 weeks, consisting of fructose : glucose in a 60 : 40 ratio. Liver fat content was measured by transient elastography through the controlled attenuation parameter (CAP) score. At baseline, the CAP score was 212.5 ± 10.1 dB m^{-1} and was not significantly different between sexes. We genotyped ten NAFLD-related SNPs, of which rs1227756 in *COL13A1* (β = −22.4 ± 7.5, $p < 0.05$) was associated with the baseline CAP score. Individuals carrying the AA allele had significantly higher CAP scores than those carrying GG (234 ± 34.7 dB m^{-1} *vs.* 188 ± 25.3 dB m^{-1}). The CAP score decreased post-SSB intervention, and the change was significantly associated with rs2228603 in *NCAN* (β = −20.1 ± 7.6, $p < 0.05$). The T-allele carriers showed a greater reduction in the CAP score as compared to CC carriers (mean ± SE −23.3 ± 5.8 dB m^{-1} *vs.* −18.24 ± 43.2 dB m^{-1}). This change was, however, not observed when adjusted for age, sex and body composition. Significant associations were also observed between changes in serum HDL and rs1260326 in *GCKR* and triglycerides and rs58542926 in *TMS6F2*. This pilot study shows a potential role of genetics in liver fat changes and serum lipids in response to SSB intervention that warrants a detailed investigation in a larger sample for a longer duration.

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Introduction

Non-communicable diseases like cancer, obesity, diabetes, cardiovascular diseases (CVD) and non-alcoholic fatty liver disease (NAFLD) have become a significant public health concern.[1–7] NAFLD, representing a group of disorders including steatosis[2,8] and non-alcoholic steatohepatitis with fibrosis,[9,10] has substantially risen in prevalence over the last two decades with an estimated prevalence of 20% among US adults and 25% in young adults.[11–13] Over 64 million individuals are believed to have NAFLD with annual medical costs rising to more than $100 billion.[14–16] NAFLD is more commonly observed in individuals who have obesity or diabetes and/or have metabolic syndrome and has been associated with increased cirrhosis, liver-related mortality and hepatocellular carcinoma.[17–19]

An unhealthy diet plays a major role in the development of NAFLD.[20–22] Fructose, present in soft drinks, fruit juices and energy drinks, affects many metabolic processes, foremost being an increase in fat accumulation in the liver[23–25] and contributing to the onset and progression of NAFLD.[26–29] Fructose is almost entirely metabolized in the liver and is rapidly phosphorylated to fructose 1 phosphate by ketohexokinase (KHK) with ATP depletion in parallel.[30,31] Fructose 1-phosphate is metabolized to dihydroxyacetone-phosphate and glyceraldehyde and finally to triglycerides,[3–5] which are deposited in the liver and lead to NAFLD.[29,30,32–34] Although both glucose and fructose affect fat accumulation in the liver, fructose seems to

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be a more potent stimulator of *de novo* lipogenesis (DNL) than glucose.[23,35,36] In population studies, it has been shown that fructose, and not glucose, is associated with increased visceral adiposity,[37–40] insulin resistance[26,37,39,40] and hyperuricemia.[41–43] Fructose is unique in its effects, as it stimulates KHK and thus potentiates its own metabolism. Second, the rate of phosphorylation of fructose by KHK is 10 times higher than the phosphorylation of glucose by glucokinase.[44,45] Third, fructose is directly absorbed into the portal vein and delivered to the liver without entering the systemic circulation.[30,44] Because of this, it passes through the liver and is exposed to a much higher fructose load than other tissues. Fourth, fructose activates lipogenic transcription factors SREBP1c[46–48] and ChREBP[49–51] in the liver, promoting DNL. Finally, fructose tends to deplete liver ATP levels, and one of the outcomes is the generation of more AMP, which is converted to uric acid.[23,30,31] Uric acid has been shown to stimulate fat synthesis in hepatocytes, thus pointing to an additional pathway through which fructose can increase liver fat content.[30,31]

In addition to diet, genetic factors contribute to the onset and progression of NAFLD.[52,53] NAFLD is a complex and heritable phenotype. Family-based studies have reported heritability estimates for NAFLD to be between 20% and 70%[54–57] and genome-wide and candidate gene studies have identified several genes associated with NAFLD.[58,59] Few studies have shown that fructose affects lipogenesis in the liver in a genotype-specific manner. Davis and colleagues found that Hispanic children with GG genotypes of the *PNPLA3* SNP rs738409 were more inclined to accumulate fat in the liver as compared to children with CC or CG genotypes.[58] Similarly, another study investigating the effects of added sugars on liver fat found that individuals with TT genotypes of rs1260326 of *GCKR* increased their *de novo* lipogenesis by 44% during an oral fructose + glucose challenge.[59] The aim of our study was to determine the role of key NAFLD-related single-nucleotide polymorphisms (SNPs) in liver fat in response to a 3-week sugar-sweetened beverage (SSB) intake in adolescents and young adults.

Experimental

Study design

This pilot open-label trial was conducted at the University of North Carolina at Chapel Hill Nutrition Research Institute (UNC-NRI). The review protocol is available at clinicaltrials.gov (# NCT03783195). A total of 72 participants aged 12 to 40 were screened. Inclusion criteria included ages between 12–40 years, no history of alcohol abuse (>7 drinks per week for a year), fructose intake of <14 drinks per week and Caucasian ethnicity. Both ethnicity and race affect the deposition of fat in the liver.[60] Studies have shown that the tendency to accumulate fat in the liver is higher in Asians and Hispanics as compared to White or Black Americans.[61] To avoid differences in liver fat content changes that may be due to ethnic differences, we focused only on one ethnic group (Caucasians) in this pilot study. Exclusion criteria included ages <12 and >40 years, pregnancy/lactation, known alcohol abuse or fructose intake >14 drinks per week, not of Caucasian ethnicity, glucose levels >100 mg dl^{-1} if fasting, >140 mg dl^{-1} if within 2 hours post meal and >200 mg dl^{-1} in a random sample, taking anti-hypertensive, anti-diabetic, uric acid- and/or lipid-lowering medications, known diagnosis of diabetes, fructose intolerance, chronic kidney disease, NAFLD or any liver-related diseases, hypertriglyceridemia, polycystic ovary syndrome, hypothyroidism, obstructive sleep apnea, hypopituitarism and hypogonadism, and liver fat fraction >5% as per baseline MRI scan. We excluded 54 participants after screening for meeting the exclusion criteria, 2 participants dropped out due to taste issues, and 1 participant was excluded after the baseline liver MRI scan due to a liver fat fraction greater than 5%. The mean age and BMI of the three participants who dropped out after visit 1 were 30 years and 27.5 kg m^{-2}, respectively. All participants provided written informed consent to the study and its procedures. The study was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill (IRB # 17-3348).

15 participants completed the 3-week study. The study was divided into two visits. Both visits, spaced three weeks apart, followed the same procedure (Fig. 1). Visit 1 and 2: following a 12-hour overnight fast, participants arrived at UNC-NRI. They were given a standardized meal for dinner the previous night and were asked to refrain from drinking. Participants collected their 24-hr urine samples the day prior to their visit and returned the samples at their visit. After signing the consent form, the participants had their anthropometrics measured. The weight measures of the subjects were taken standing while wearing shoes and light clothing. A stadiometer against the wall was used to measure height, in an upright standing position, to the closest 0.1 cm. Their body mass index (BMI kg m^{-2}) was calculated using the height and weight values. A stretch-resistant tape close to the umbilical region was measured for waist circumference (WC) to the nearest 0.1 inch. Bioelectrical impedance analysis (BIA) using a Tanita Dual Frequency Total Body Composition Analyzer (DC-430U, Tokyo, Japan) was used to record every subject's body composition. All anthropometric measurements were taken by the same staff member to minimize measurement variation and margin of error. An Omron digital blood pressure monitor (HEM907XL, Omron Healthcare Inc., Lake Forest, IL, USA) was used on the right arm of the subjects to measure blood pressure. Two measurements of BP were taken with an interval of 1 min; the average was calculated and used for statistical analysis. A detailed questionnaire on medical and dietary data was administered to the participants on the day of the visit. Participants were then provided individually packaged packets of powder containing 0.75 g per kg body weight of fructose + 0.45 g per kg body weight of glucose which approximates the 60 : 40 ratio found in regular sodas.[62] Participants were instructed to consume the packets of fructose dissolved in 24 oz of water and consume the drink daily for the three-week intervention period. They were also instructed not to consume

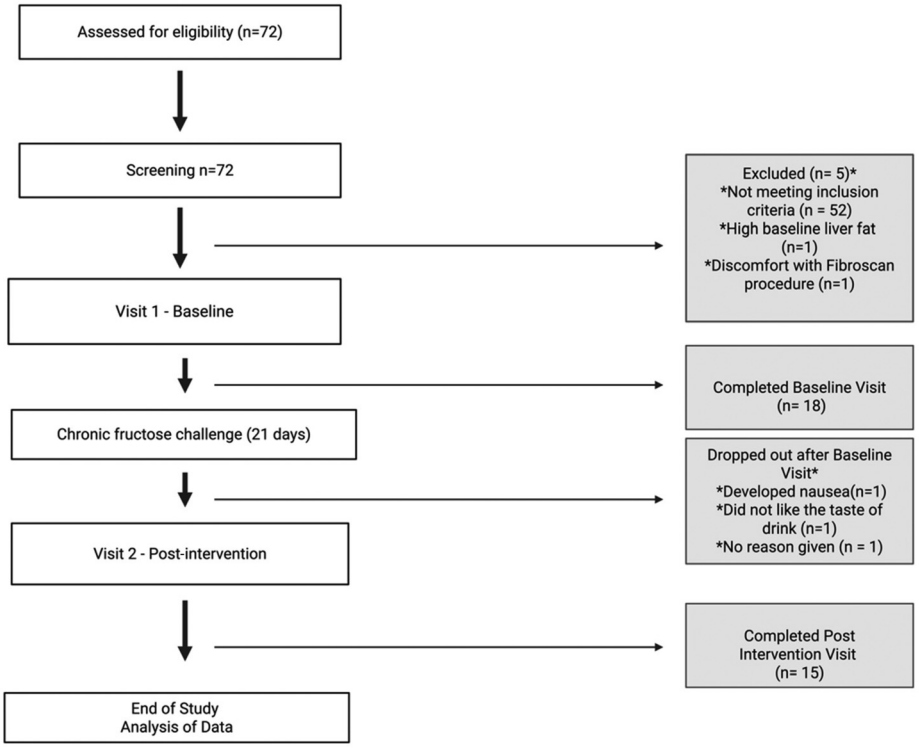

Fig. 1 Consort diagram of the intervention study. Image created with BioRender.

any other SSBs during the 3-week period. All participants brought back empty packets for compliance checking.

SNP selection for genotyping

Ten single nucleotide polymorphisms (SNPs) were selected for genotyping based on their association with NAFLD or liver fat from previous published studies: rs12137855 in *LYPLAL1*, for its influence on triglyceride lipase in adipose tissue and depalmitoylating calcium-activated potassium channels,[63] rs1227756 in *COL13A1*, for its association with connective tissue proliferation[64] and liver injury,[65] the missense variant rs738409 in *PNPLA3* for its role in energy usage/storage in adipocytes,[63,64] rs887304 in the *EFCAB4B* gene for its role in calcium binding and regulation,[65] *GCKR* rs1260326 for its involvement in hepatic fat accumulation along with large very low density lipoprotein (VLDL) and triglyceride levels (Fig. 2),[63,66] the missense variant in *APOC3* gene rs2854116 for its role in hypertriglyceridemia and impact of dietary fat intake and NAFLD,[67,68] rs2645424 in *FDFT1* for its role in cholesterol biosynthesis,[69] *TMS6F2* gene variant, rs58542926 for its role in post-prandial lipemia,[70,71] rs2228603 in the *NCAN* gene for its association with increased risk of liver inflammation and fibrosis,[72] and rs4240624 in the *PPP1R3B* gene for its association with glycogen metabolism.[73,74] The SNP positions and their frequencies are shown in Table 1.

Genotyping

DNA was extracted from saliva *via* an automated nucleic acid extraction platform (Anaprep 12, Biochain, Institute Inc., Newark, CA, USA) using the Anaprep Forensic DNA extraction kit (Biochain Institute Inc., Newark, CA, USA). The concentration and purity of genomic DNA were measured using a NanoDrop spectrophotometer. Genotyping was performed *via* TaqMan® predesigned SNP genotyping assay (Applied Biosystems, Foster City, CA, USA) on a QuantStudio 12k Flex real-time PCR system (Thermo Fisher Scientific, USA).

Liver fat content and stiffness measurement

Transient elastography using FibroScan® (Echosens, Netherlands) was used to assess fat content in the liver and liver stiffness.[75,76] FibrosSan measurements were performed at both visits. This is a non-invasive measure that uses ultrasound technology to measure liver stiffness and adiposity. The CAP score, which is measured in decibels per meter (dB m^{-1}), is used to grade liver steatosis. A score below 238 dB m^{-1} is considered normal. Scores between 238 dB m^{-1} and 260 dB m^{-1} indicate grade 1 (S1) steatosis. Scores between 260 dB m^{-1} and 290 dB m^{-1} indicate S2, and scores between 290 dB m^{-1} and 400 dB m^{-1} indicate S3. As for Emed values, scores between 2 kPa and 7 kPa (F0–F1) are normal, a score between 7.5 kPa and 10 kPa (F2) is considered moderate scarring, a score between 10 kPa and 14 kPa (F3) is considered severe scarring, and a score of 14 kPa or higher (F4) indicates cirrhosis. In this study, the CAP score was used to indicate the amount of fat in the liver.[82,83]

Sample collection, processing and storage

At both visits, fasting blood and 24-h urine samples were collected. A trained phlebotomist collected blood through venous puncture using 6 mL ethylenediaminetetraacetic acid (EDTA)-

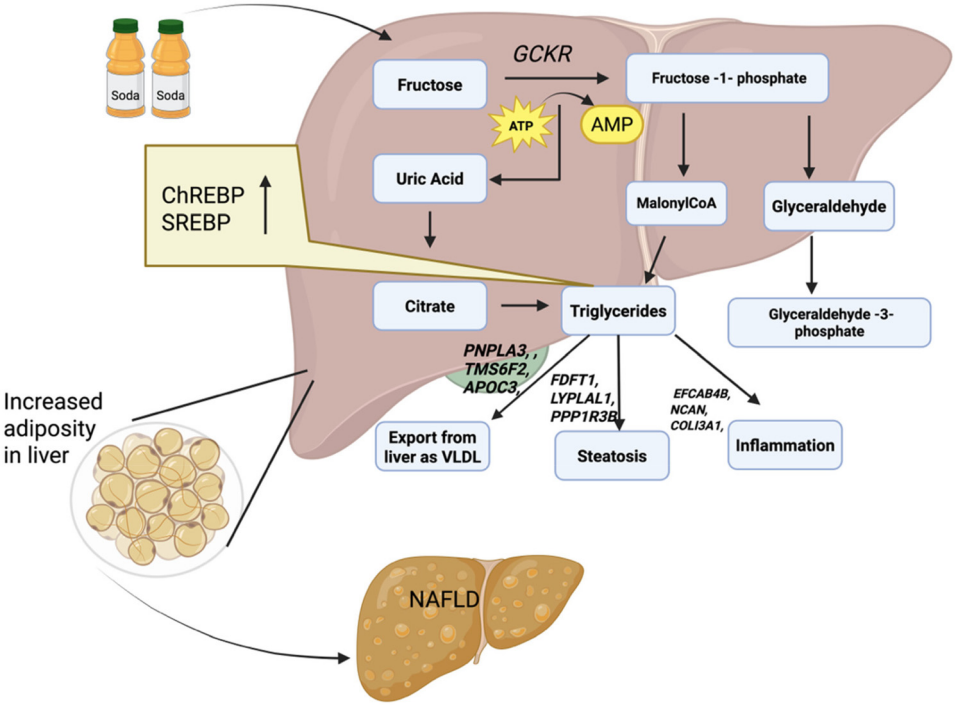

Fig. 2 Mechanism of dietary sugars metabolism and liver fat accumulation. ATP – adenosine triphosphate; AMP – adenosine monophosphate; *GCKR* – glucokinase regulatory protein; ChREBP – carbohydrate response element binding protein; SREBP – sterol regulatory element binding protein; *PNPLA3* – patatin-like phospholipase domain-containing protein 3; *TM6SF2* – transmembrane 6 superfamily member 2; *APOC3* – apolipoprotein C-III; *FDFT1* – farnesyl-diphosphate farnesyltransferase 1; *LYPLAL1* – lysophospholipase-like1; *PPP1R3B* – protein phosphatase 1 regulatory subunit 3B; *EFCAB4B* – *EF* hand calcium-binding domain-containing protein 4B; *NCAN* – neurocan; and *COLI3A1* – collagen type III alpha 1 chain. Image created with BioRender.

Table 1 SNPs selected for genotyping

SNP	Gene	Risk allele	SNP position	Allele frequency in general population (Ensembl)	Allele frequency in our study sample
rs12137855	*LYPLAL1*: intergenic variant	C	1: 219275036	0.84	0.79
rs1227756	*COL13A1*: intron variant	G	10: 69828748	0.61	0.43
rs738409	*PNPLA3*: missense variant	G	22: 43928847	0.26	0.27
rs887304	*EFCAB4B*: 3′ UTR variant	T	12: 3648382	0.14	0.39
rs1260326	*GCKR*: missense variant	T	2: 27508073	0.29	0.43
rs2854116	*APOC3*: regulatory region variant	C	11: 116829453	0.55	0.47
rs2645424	*FDFT1*: intron variant	A	8: 11826954	0.52	0.30
rs58542926	*TMS6F2*: stop gained variant	C	19: 19268740	0.93	0.77
rs2228603	*NCAN*: missense variant	T	19: 19219115	0.04	0.13
rs4240624	*PPP1R3B*: intron variant	A	8: 9326721	0.89	0.87

coated tubes and serum tubes (BD Vacutainer, Becton, Dickinson & Company, Franklin Lakes, NJ, USA). Immediately after collection, EDTA tubes were placed on wet ice and centrifuged at 3000 RPM for 15 min at 4 °C. Serum, plasma, buffy coat and urine samples were aliquoted and stored at −80 °C for further biochemical measurements.

Biomarker measurements

At each visit, serum concentrations of lipids, total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) and triglycerides were measured using fluorometric assays (Abcam, Cambridge, MA, USA). Uric acid concentration in serum was measured using fluorometric assays, as per the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA) on a BioTek Synergy 2 Multi-Mode plate reader (BioTek, Winooski, VT, USA). All measurements were conducted in duplicate, and the coefficient of variation was less than 10%.

Statistical analysis

Statistical analyses were performed using STATA software version 19.5 (College Station, TX, USA). The primary outcomes of this study were CAP and Emed scores, and the secondary outcomes were body composition measurements (BMI, waist circumference, waist–height ratio, percent body fat, fat mass and fat-free mass),

and serum concentrations of lipids (total cholesterol, HDL, VLDL, LDL cholesterol and triglycerides) and uric acid. As a first step, paired *t*-tests were conducted to determine whether there were any significant changes in variables pre- and post-intervention. Linear regression analyses were performed to determine the association between the ten selected SNPs and baseline values and changes in body composition, liver fat content and serum concentrations of lipids and uric acid. Baseline values or changes in liver fat content and other biomarkers were considered as outcome or dependent variables, and SNPs were considered as the predictor or independent variables. All analyses were adjusted for sex, age and baseline concentrations of the variables and waist-height ratio wherever applicable. All results were considered significant at $p < 0.05$.

Results

The descriptive characteristics of the participants are listed in Table 2. Fifteen participants, 5 males ($n = 5$) and 10 females ($n = 10$), participated and completed the 3-week SSB intervention. Their mean BMI and age were 25.03 ± 1.1 kg m^{-2} and 25.53 ± 2.4 years, respectively. At the baseline, significant differences were observed between females and males with respect to fat-free mass (104.8 ± 3.8 *vs.* 137.7 ± 8 11.2 g, $p < 0.05$) and percent body fat (31.5 ± 2.0 *vs.* 17.0 ± 3.0%, $p < 0.05$). Serum urate concentrations were significantly higher in males than in females (7.35 ± 0.4 *vs.* 6.1 ± 0.3 mg dl^{-1}, $p < 0.05$). Baseline CAP score was significantly associated with fat mass ($\beta = 1.2 \pm 0.5$, $p < 0.05$), waist circumference ($\beta = 2.3 \pm 0.9$, $p < 0.05$), and systolic blood pressure ($\beta = 1.6 \pm 0.4$, $p < 0.005$).

A paired *t*-test was conducted to detect differences between pre- and post-intervention anthropometrics, liver fat content, and serum biomarker values (Table 3). Most of the biomarkers showed no changes post-intervention. The CAP score, however, showed a decreasing, albeit statistically insignificant, trend ($p = 0.07$), contrary to our hypothesis. When the data were analyzed using residuals after adjusting for age, sex and waist-height ratio, there was no difference in CAP score between pre- and post-intervention ($p = 1.00$). Similarly, we observed a decrease in serum urate concentrations after the 3-week intervention which was mitigated after adjusting for the covariates. The change in CAP score was significantly correlated with baseline waist circumference ($r^2 = 0.53$, $p < 0.05$) and not with fat mass or systolic blood pressure, as observed with baseline CAP score. Although the baseline CAP score was not correlated with the baseline waist-height ratio, the changes in the two variables were significantly correlated ($r^2 = 0.71$, $p < 0.005$). Changes in Emed score, waist circumference and waist-height ratio were also associated with their baseline values ($p < 0.05$), respectively.

The ten genotyped SNPs were selected based on their links to NAFLD. Their minor allele frequencies ranged between 13 and 47%. Genotype-specific analysis showed that rs1227756,

Table 3 Anthropometrics and biomarkers pre- and post-SSB intervention

Variable	Visit 1 (mean ± SE)	Visit 2 (mean ± SE)	*P*-value
BMI (kg m^{-2})	25.03 (1.07)	25.13 (1.05)	0.27
PBF (%)	0.27 (0.03)	0.27 (0.03)	0.16
FM (lbs)	43.86 (5.24)	44.71 (5.24)	0.15
FFM (lbs)	115.74 (5.95)	115.77 (6.15)	0.94
Waist circumference (cm)	87.92 (3.04)	88.16 (2.82)	0.88
Wc/Ht	0.45 (0.05)	0.51 (0.02)	0.16
SBP (mmHg)	124.67 (4.51)	123.07 (3.18)	0.75
DBP (mmHg)	79.47 (3.25)	79 (2.03)	0.88
Emed (kPa)	4.51 (0.25)	5.01 (0.49)	0.41
CAP score (dB m^{-1})	212.15 (10.15)	192.91 (13.57)	0.07
Triglycerides (mg dl^{-1})	62.47 (12.92)	73.07 (21.47)	0.41
HDL (mg dl^{-1})	30.98 (6.39)	29.72 (5.66)	0.79
LDL (mg dl^{-1})	130.25 (11.21)	128.35 (11.08)	0.79
Total cholesterol (mg dl^{-1})	173.73 (12.09)	172.68 (12.95)	0.92
Uric acid (mg dl^{-1})	6.50 (0.30)	6.43 (0.28)	0.04

Table 2 Descriptive characteristics of participants at the baseline

	Female ($N = 10$) Mean ± SE	Male ($N = 5$) Mean ± SE	Total ($N = 15$) Mean ± SE	*P*-value
BMI (kg m^{-2})	25.54 (1.24)	24 (2.19)	25.03 (1.07)	0.52
Age (years)	26.60 (2.81)	23.40 (4.66)	25.53 (2.37)	0.54
Percent body fat (%)	31.5 (2.0)	17.0 (3.0)	27.0 (26.0)	0.003
Fat mass (lbs)	50.29 (5.92)	31 (8.31)	43.86 (5.24)	0.08
Fat-free mass (lbs)	104.77 (3.82)	137.68 (11.20)	115.74 (5.95)	0.003
Waist circumference (cm)	87.02 (3.33)	89.36 (6.33)	87.92 (3.04)	0.73
Waist–height ratio	0.42 (0.07)	0.50 (0.03)	0.44 (0.05)	0.43
SBP (mmHg)	124.00 (5.59)	126.00 (8.50)	124.67 (4.51)	0.84
DBP (mmHg)	78.50 (4.20)	81.40 (5.49)	79.47 (3.25)	0.69
Emed (kPa)	4.46 (0.34)	4.59 (0.38)	4.51 (0.25)	0.82
CAP score (dB m^{-1})	222.23 (13.07)	192.00 (12.66)	212.15 (10.15)	0.17
Triglycerides (mg dl^{-1})	65.7 (17.18)	56 (20.24)	62.47 (12.92)	0.74
HDL (mg dl^{-1})	34.19 (8.29)	24.58 (10.17)	30.98 (6.39)	0.50
LDL (mg dl^{-1})	126.06 (13.30)	138.64 (22.32)	130.25 (11.21)	0.62
Total cholesterol (mg dl^{-1})	172.38 (15.58)	174.42 (21.02)	173.73 (12.09)	0.97
UA (mg dl^{-1})	6.07 (0.34)	7.35 (0.41)	6.49 (0.30)	0.04

Table 4 Regression analysis (change in variable as the dependent variable and SNP as the independent variable adjusted for the baseline of the variable value, gender, age and waist circumference)

Change in variable	SNP Id	Beta coefficient ± SE	*P* value*	95% CI	
Primary outcome:					
CAP_med dBm	rs58542926	22.26 (±9.61)	0.05	−0.47	44.98
	rs2228603	−20.10 (±7.59)	0.03	−38.05	−2.16
Secondary outcomes:					
Systolic blood pressure	rs12137855	−11.14 (4.33)	0.04	−21.39	−0.9
	rs1227756	0.62 (4.60)	0.05	−1.44	0.01
Diastolic blood pressure	rs12137855	−9.91 (1.80)	0.001	−14.06	−5.56
HDL	rs1260326	11.73 (4.61)	0.04	0.82	22.63
TAG	rs58542926	77.07 (26.18)	0.02	15.16	138.97
VLDL	rs58542926	15.41 (5.24)	0.02	3.03	27.79

*Results with p values < 0.06 are shown here.

an intronic variant in *COL13A1* ($\beta = -22.4 \pm 7.5$, $p < 0.05$), was associated with the baseline CAP score, with individuals carrying the AA allele having significantly higher CAP scores (234 ± 34.7 dB m^{-1}) as compared to those carrying the GG allele (188 ± 25.3 dB m^{-1}).

At the baseline, systolic blood pressure was associated with rs12137855, an intronic variant in *LYPLAL1* (β ± SE −11.14 ± 4.3, $p < 0.05$) (Table 4). The same SNP was associated with changes in diastolic blood pressure (−9.91 (±1.80), $p < 0.005$). The CAP score decreased post-SSB intervention, and the change was significantly associated with rs2228603, a missense variant in *NCAN* ($\beta = -20.1 \pm 7.6$, $p < 0.05$). T allele carriers showed a greater reduction in CAP score as compared to CC carriers (mean ± SE −23.3 ± 5.8 dB m^{-1} *vs.* 18.24 ± 43.2 dB m^{-1}). Significant associations were also observed between changes in serum HDL and rs1260326, a missense variant in *GCKR* (β ± SE 11.73 ± 4.6, $p < 0.05$), and between triglycerides and rs58542926, a stop-gained variant in *TM6SF2* (β ± SE 77.1 ± 26.2, $p < 0.05$).

Discussion

The main aim of this study was to determine whether SNPs previously linked with NAFLD tend to associate with the CAP score at the baseline and the CAP score's response to SSB intake. In this study, we utilized transient elastography FibroScan, a non-invasive assessment of liver stiffness/fibrosis (Emed) and steatosis (CAP score). These measures have increasingly been used for the evaluation of patients with NAFLD and NASH over liver biopsy.[77–80] The CAP scores present the total attenuation of sound waves, an indirect measure of steatosis.[81–84] However, in this study, we used CAP scores as a surrogate measure of liver fat content as reported in some previous studies.[85,86] We found that the CAP score at the baseline was positively associated with body composition and systolic blood pressure. Similar results have been shown by other studies.[87–89] In a study in NAFLD patients, the CAP score was positively correlated with fat mass, waist circumference and waist–height ratio.[87] In another study of patients with overweight and obesity, CAP values were positively associated with fat mass, BMI and homeostasis model assessment of insulin resistance (HOMA-IR).[88] Another study in youths found that individuals with higher CAP scores had higher BMI, waist circumference and other fat distribution measures.[85]

A CAP score below 238 dB m^{-1} is considered normal liver fat content.[90] In our study, both males and females had CAP scores lower than 238 dB m^{-1}. We hypothesized that the three-week SSB intervention will increase the liver fat content. In contrast, the CAP scores decreased slightly after the 3-week period. The non-alcohol-related fat deposition in the liver is dependent on an individual's age, sex, dietary intake of simple sugars and saturated fats, physical activity and the presence of other metabolic disorders such as obesity, insulin resistance and metabolic syndrome.[91–94] In our study, we think that the decrease in the CAP score may be due to many reasons: participants' low CAP scores at the baseline, our stringent inclusion criteria with the exclusion of adverse metabolic conditions, the young age of the participants, and the participants' activities and healthy lifestyles. This may also explain why there was no change in the CAP score when the data were adjusted for age, sex and body composition. Although we had advised the participants not to alter any of their dietary habits, except for reducing SSB intake, it is possible that the participants may have cut down on their other sources of sugar. Studies under controlled or domicile conditions may reflect the true effect of simple sugars on liver fat content.

The Emed score is a measure of fibrosis/liver stiffness, where higher values indicate higher levels of liver stiffness or liver tissue scarring. In our study, Emed scores were very similar between males and females, and we observed an increase in the mean scores after the fructose intervention, which was negligible and non-significant. Similar to the liver physiological measures, anthropometrics and body composition measures such as percent body fat and fat mass showed no significant changes. There were significant differences in percent body fat and fat mass measures between males and females at the baseline. Studies have shown that the response to fructose intervention differs between males and females.[95,96] Males are usually reported to have adverse metabolic effects such as insulin resistance, high blood pressure and hyperlipidemia in response to increased fructose intake.[96–98] Females, although higher in fat mass and percent body fat, tend not to have higher metabolic

adversities compared to males.[96,97] This may be due to the role of hormones such as estrogen in lipid metabolism and storage.[99]

Fructose metabolism inherently generates uric acid,[41] and hyperuricemia has been linked to the onset and progression of NAFLD.[100–102] In a previous fructose response study in our lab, we found that fructose intervention caused serum uric acid levels to spike and did not return to the baseline levels until 150 minutes after the intake of fructose.[42,43] In this trial, we wanted to understand the long-term effects of SSBs on adiposity and uric acid. Examination of the effects of 3-week fructose exposure on serum urate levels in this study did not show any significant change post-intervention compared to the baseline. However, we did find that males had higher serum uric acid levels than females at the baseline, which is consistent with other studies.[42,43] This finding again highlights the differences in uric acid metabolism in males and females, highlighting protective effects against hyperuricemia in pre-menopausal females.[103,104]

In addition to sex-specific differences, interindividual variability – particularly genetic variation – affects hepatic lipid responses to fructose intake.[105] In this study, we explored and found few potential links between NAFLD-related SNPs and liver fat content and serum lipids. The SNP rs12137855 in the *LYPLAL1* gene was related to systolic blood pressure especially the homozygous T genotype. This gene has been studied for metabolic traits such as fat distribution, obesity, and hypertension.[63,106] Other SNPs, rs2605100 and rs4846567, in the same gene, *LYPLAL1*, have been found to be associated with the above-mentioned phenotypes and appetite suppression in a Japanese population.[107,108] Another SNP, rs2605100 in *LYPLAL1*, was found to be associated with high blood pressure in a cohort of Chinese children.[109] These findings suggest that *LYPLAL1* has several variants that could influence blood pressure and thereby cardiovascular function.

SNP rs1227756 in the *COL13A1* gene was associated with increased systolic blood pressure (A/A genotype). This SNP has been studied in relation to lobular inflammation in Caucasian women.[110] SNP rs2854116 in the *APOC3* gene has been significantly associated with decreased diastolic blood pressure, especially in individuals with the homozygous C genotype. Although this gene has traditionally been studied for its role in liver fat and dyslipidaemia,[111–113] our results suggest a novel role in blood pressure regulation.

The other interesting link we found was between SNP rs58542926 and CAP values. Individuals with the T allele of rs58542926 in the *TM6SF2* gene were observed to have lower CAP values. In a previous study conducted in a Han Chinese population, the same allele was associated with increased CAP scores and an increased risk of NAFLD.[114] However, in another meta-analysis study across different populations, including Chinese, the T allele of rs58542926 was associated with a lower lipid profile and a protective effect against CVD risk.[115] Combined with findings from our study which is 100% Caucasian, the SNP seems to take on a dual and opposing role depending on the ethnicity of the study population.

Individuals with the homozygous C allele and homozygous T allele of rs2228603 have been observed to have lower CAP values. This SNP is in the *NCAN* gene and has shown a strong association with the CAP score, with the T allele being linked to a greater reduction in the CAP score. In a previous study that consisted of the European descent Caucasian and Old Order Amish population, the SNP's T allele was associated with an increased risk of hepatic fat accumulation.[116] In another study involving a Chinese population, the same allele was associated with high levels of HDL and also increased levels of alkaline phosphatase, showing opposing dual effects.[117] Interestingly, in a study conducted in an 80% female bariatric patient cohort, rs2228603 T was associated with an increased risk of steatosis.[72]

We also found associations between genetic variants and changes in serum HDL and triglycerides. The SNP rs1260326 in *GCKR* was associated with HDL levels in our study. In a study on children, this SNP was associated with liver fat content as measured by MRI.[118] Most studies have reported T allele carriers to have lower levels of HDL which is in contrast to what we observed in our study.[118–120] We found that T allele carriers had higher HDL levels than CC carriers. We found similar differences with respect to the association of rs58542926 in *TM6SF2* with serum triglycerides. Other studies have reported that the T allele is associated with lower triglyceride levels.[121,122] Although we found higher concentrations in T carriers at the baseline, their triglyceride levels decreased after the 3-week SSB intervention, while CC carriers showed an increase in their triglyceride levels. This indicates that the T allele may have a protective effect on serum triglycerides in our sample. The SNP rs738409 in *PNPLA3* has been linked to liver fat in many studies, but we did not find its association with either the baseline levels or their changes.

There are a few limitations to the study that should be addressed in future studies. First, the sample size of 15 is small for a clinical trial. Second, each genotype group was limited to 3–5 participants, and third, the 3-week duration may be too short to observe significant changes in lipid profiles such as LDL-C, HDL-C, and total cholesterol. It is also possible that short-term fructose exposure might cause biochemical changes in hepatocytes without reaching the threshold for measurable fat accumulation. Additionally, anthropometric changes such as weight gain and fat redistribution typically require more than 3 weeks to show significant changes. Underlying hormonal changes such as leptin, adiponectin, cortisol and other metabolic hormones do not significantly shift within the limited duration of SSB intervention. Furthermore, the dosage of fructose might be too low to elicit measurable changes within this timeframe, as our dose is equivalent to two 12-ounce soda cans. The results may not be generalizable across all ethnic groups. Moreover, all participants had low or normal CAP scores at the baseline (non-steatotic range), which may limit the sensitivity of the CAP score. However, the key strengths of the study are its stringent inclusion criteria, a homogeneous sample, and that it is one of the very few studies that studied the associations between

genetic variants and changes in the CAP score in response to an SSB intervention in an ethnically homogeneous population.

Conclusions

In summary, a 3-week SSB intervention did not affect the liver fat content or the liver fat markers in our young adult population. Genetic heterogeneity is another important puzzle piece of NAFLD to understand individual variability in disease susceptibility and progression. This study unlocks the possible role of SNPs that may influence the NAFLD onset and progression to better understand the role of genetics in this disease. It also provides pilot data for conducting larger studies where genetically susceptible groups could be identified, and their responses to nutrient intake can be measured. Together, this could implement novel treatment, management and prevention strategies for NAFLD.

Abbreviations

ACC	Acetyl CoA carboxylase
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AMP	Adenosine monophosphate
APOC3	Apolipoprotein C-III
AST	Aspartate transaminase
ATP	Adenosine triphosphate
BMI	Body mass index
CAP	Controlled attenuation parameter
ChREBP	Carbohydrate response element-binding protein
COLI3A1	Collagen type III alpha 1 chain
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DNL	*De novo* lipogenesis
EFCAB4B	EF hand calcium-binding domain-containing protein 4B
FAS	Fatty acid synthase
FDFT1	Farnesyl-diphosphate farnesyltransferase 1
FLFS	Fructose liver fat study
GCKR	Glucokinase regulatory protein
GGT	Gamma-glutamyl transferase
GLUT 2	Glucose transporter protein 2
GLUT 4	Glucose transporter protein 4
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
LYPLAL1	Lysophospholipase-like1
NAFLD	Nonalcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCAN	Neurocan
PNPLA3	Patatin-like phospholipase domain-containing protein 3
PPP1R3B	Protein phosphatase 1 regulatory subunit 3B
PWV	Pulse wave velocity
SBP	Systolic blood pressure
SNP	Single-nucleotide polymorphism
SREBP	Sterol regulatory element-binding protein
TAG	Triacylglycerols
TC	Total cholesterol
TM6SF2	Transmembrane 6 superfamily member 2
UA	Uric acid
VLDL	Very low-density lipoprotein
WC	Waist circumference
MAFLD	Metabolic dysfunction-associated fatty liver disease
KHK	Ketohexokinase
SSB	Sugar-sweetened beverage

Author contributions

Conceptualization, VSV; methodology, FTJ, KN, KW, LRG, BCN, and BBM; software, SSV, FTJ, and VSV; formal analysis, SSV, FTJ, and VSV; investigation, FTJ, SSV, and VSV; resources, VSV; writing – original draft preparation, FTJ, SSV, and VSV; writing – review and editing, FTJ, SSV, LRG, and VSV; project administration, VSV; and funding acquisition, VSV. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

Data availability

Data supporting the findings of this study are not publicly available due to privacy reasons but are available from the corresponding author upon reasonable request.

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