

## **NAFLD exacerbates the effect of dietary sugar on liver fat and development of an atherogenic lipoprotein phenotype**

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**Abstract word count:** 258

**Word count:** 3,844

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## **Abstract**

**Aims/hypothesis:** We aimed to test the hypothesis that the effects of dietary sugar on lipoprotein metabolism are influenced by non-alcoholic fatty liver disease (NAFLD).

**Methods:** The effect of two 12 week, iso-energetic diets, high and low in non-milk extrinsic sugars (26% and 6% total energy), matched for macronutrient content, was examined in a randomised, cross-over study in men with NAFLD (n=11) and controls (n= 14). Lipoprotein kinetics and the sources of fatty acids for triacylglycerol (TAG) production were measured using stable isotope tracers.

**Results:** Liver fat was higher after the high versus low-sugar diet in both groups ( $p<0.02$ ), but men with NAFLD showed a relatively greater response than controls ( $p<0.05$ ). After the high versus low-sugar diet, VLDL<sub>1</sub>-TAG production rate was higher in the controls ( $p <0.002$ ) due to a greater contribution from splanchnic fatty acids ( $p<0.02$ ) and de novo lipogenesis ( $p <0.002$ ), whereas in NAFLD, VLDL<sub>2</sub>-TAG production rate was higher ( $p <0.05$ ), due to a greater contribution from splanchnic fatty acids ( $p<0.02$ ). There was no difference in the contribution of systemic NEFA to VLDL<sub>1</sub> and VLDL<sub>2</sub>-TAG production rate between diets in either group. Intermediate density lipoprotein (IDL), LDL<sub>2</sub> and LDL<sub>3</sub>-apolipoprotein B production rates and post-heparin hepatic lipase activity were all higher ( $p<0.05$ ) on the high-sugar diet in NAFLD.

**Conclusions:** A high sugar intake promoted a greater accumulation of liver fat in NAFLD than controls and increased VLDL-TAG production in both groups, due mainly to an increased contribution of fatty acids from splanchnic sources, which includes hepatic TAG storage pools. These effects may drive the formation of atherogenic lipoproteins.

**Clinical Trial Registration:** NCT01790984

**Key words:** Sugar, Triacylglycerol, NAFLD, De novo lipogenesis, Stable isotopes, VLDL, LDL, NEFA, hepatic lipase, kinetics

**Abbreviations:** apolipoprotein (apo), atherogenic lipoprotein phenotype (ALP), de novo lipogenesis (DNL), fractional catabolic rate (FCR), gas chromatography mass spectrometry (GCMS), hepatic lipase (HL), Intermediate density lipoprotein (IDL), intra-hepatocellular lipid (IHCL), lipoprotein lipase (LPL), magnetic resonance imaging (MRI) and spectroscopy (MRS), non-alcoholic fatty liver disease (NAFLD), National Diet & Nutrition Survey (NDNS), non-milk extrinsic sugars (NMES), tracer/tracee ratio (TTR), production rate (PR), small dense low density lipoprotein (sdLDL), steady state (SS),

## **Introduction**

A high intake of dietary free sugars, mainly as sugar added to food, can increase cardiovascular risk by promoting adverse changes in plasma lipoproteins, known collectively as an atherogenic lipoprotein phenotype (ALP) [1, 2]. This may be influenced by the presence of NAFLD which is associated with hypertriglyceridaemia and insulin resistance [3].

An elevated plasma concentration of TAG is a pre-requisite for the development of an ALP via the remodelling of LDL into small and dense particles with increased potential to promote atherosclerosis [4]. Plasma TAG may be increased by an overproduction and secretion of its principal transporter, VLDL from the liver, and/or impaired removal of VLDL from the plasma via the action of lipoprotein lipase (LPL) [5]. During fasting, the production of VLDL-TAG in the liver is mainly regulated by the availability of NEFA from peripheral adipose tissue (systemic sources) or splanchnic sources [6]. The latter includes visceral adipose tissue, intra-hepatic stores, and the synthesis of fatty acids by *de novo* lipogenesis (DNL) in the liver. Although DNL makes a relatively small contribution to VLDL-TAG production, this has been shown to increase substantially when a very high proportion of energy is supplied as sugar, especially sucrose and fructose [7]. However, the extent to which liver fat affects the handling of hepatic fatty acids in response to sugar intakes representative of a Western diet is unknown. This study was designed to test the hypothesis that the presence of NAFLD influences the effect of a high sugar versus a low sugar isoenergetic diet, on lipoprotein metabolism.

## **Material and Methods**

### *Participants*

Exclusion criteria were diabetes and any medical condition other than NAFLD, lipid-lowering medication, unstable weight in the preceding 3 months, and an intake of alcohol exceeding 20g/day. Inclusion criteria were men aged 40-65y, BMI 25-30 with raised cardiovascular risk, as assessed by a risk score used previously in the 'RISCK' study [8]. Those with a raised metabolic score of  $\geq 4$  and *APO ε3/ε3* genotype, to exclude the confounding effects of different apo E isoforms on lipid metabolism, underwent an assessment of intrahepatocellular lipid (IHCL) by magnetic resonance spectroscopy (MRS) for assignment to a group with NAFLD [9] ( $>5.56\%$  IHCL, n=11) or low liver fat (Controls) ( $<5.56\%$  IHCL, n=14). All participants provided written informed consent before taking part in the study. The study was approved by Surrey Research Ethics Committee (Ref. 08/H1109/227), and University of Surrey's Ethics Committee (Ref. EC/2009/29) and was registered on Clinical Trials.gov (NCT01790984).

### *Study design, dietary interventions and study protocol*

The study had a randomized two-way crossover design, with two dietary interventions. After an initial 4 week run-in period on their habitual diet, participants were randomly assigned (with a computer-generated sequence of treatments concealed in sealed envelopes) to either the high or low sugar isoenergetic diet, with the same macronutrient composition, for 12 weeks. Participants returned to their habitual diet for 4 weeks, before crossing-over to the alternative diet for a further 12 weeks. During the dietary interventions, participants were instructed to maintain their habitual level of physical activity. Body fat, liver fat, lipoprotein and lipid kinetics were measured at the end of each diet.

Intakes of total carbohydrate and sugar were based on the mean intakes for men aged 40-65 years in the UK's National Diet & Nutrition Survey (NDNS), with target intakes for non-milk extrinsic sugars (NMES) on the high and low sugar diets corresponding to the upper and lower 2.5<sup>th</sup> percentile of intake in the UK population, respectively [10]. The term NMES, as originally defined by the UK's Department of Health [11], includes free sugars added to food, but excludes sugar in whole fruit, and lactose, primarily from cows' milk [12]. The sugar content of the two diets was achieved by a dietary exchange of sugar for starch as described in 'Supplementary material'. Dietary intakes assessed by 3-day diet diaries, (2 weekdays and 1 weekend day during the run-in period and during the sixth and final week of each dietary intervention), were analysed by a single operator using *DietPlan 6* (version 6.50, Forestfield Software Ltd, UK).

#### *Metabolic study (Post-diet visits)*

The study design is shown in Supplementary Figure 1. The evening before the metabolic study subjects drank <sup>2</sup>H<sub>2</sub>O (3g/kg body water, half after the evening meal (standardised low fat, low fibre ready-meal) and half at 10pm). They then fasted and drank only water enriched with <sup>2</sup>H<sub>2</sub>O (4.5g <sup>2</sup>H<sub>2</sub>O/liter drinking water). The following morning a blood sample was taken to measure deuterium enrichment of palmitate in VLDL<sub>1</sub> and VLDL<sub>2</sub>-TAG and plasma water to measure DNL. A 10h primed constant iv [1-<sup>13</sup>C]leucine infusion (1mg/kg; 1mg/kg/h) (99%, Cambridge Isotopes) was administered to measure VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL, LDL<sub>2</sub> and LDL<sub>3</sub>-apoB kinetics. An 8h constant iv infusion of [U-<sup>13</sup>C]palmitate (99%, Cambridge Isotopes) bound to human albumin (5%, 0.01 μmol.kg<sup>-1</sup>.min<sup>-1</sup>), was administered to measure palmitate production rate (assumed to be mainly from systemic adipose tissue lipolysis) and the percentage contribution of systemic NEFA to triglyceride export in VLDL<sub>1</sub> and VLDL<sub>2</sub>. An intravenous bolus of [1,1,2,3,3-<sup>2</sup>H<sub>5</sub>]glycerol (75μmol/kg) (99%, Cambridge Isotopes) was administered to measure VLDL<sub>1</sub> and VLDL<sub>2</sub>-TAG production rate (PR). At

varying time intervals as previously reported [13, 14], blood samples were taken to measure the enrichment and concentrations of plasma palmitate,  $\alpha$ ketoisocaproate ( $\alpha$ KIC) and glycerol, and the enrichment and concentrations of apoB, TAG-palmitate and TAG-glycerol in the lipoprotein fractions. At the end of the study, the activity of lipoprotein lipase (LPL) and hepatic lipase (HL) were measured in plasma, before and 15 minutes after an intravenous injection of 50U/kg heparin.

#### *Magnetic resonance imaging (MRI) and spectroscopy (MRS)*

Whole body MR images were obtained on a 1.5T Phillips Achieva system (Philips Medical Systems, Best, The Netherlands). Volumes of intra-abdominal and subcutaneous abdominal adipose tissue were calculated from the abdominal region between the slice containing the bottom of the lungs/top of the liver and the slice containing the femoral heads. Spectra were analyzed by a single trained observer (ELT) using AMARES. Liver fat was measured relative to liver water content as previously described [15]. Seventeen of the 25 participants who completed both diets underwent a post-dietary analysis of IHCL and adipose tissue distribution by MRS.

#### *Laboratory methods*

VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL, LDL<sub>2</sub> and LDL<sub>3</sub> were separated by sequential ultracentrifugation [16]. Plasma TAG, VLDL<sub>1</sub> and VLDL<sub>2</sub>-TAG were extracted and isotopic enrichment of glycerol and palmitate measured as described previously by gas chromatography mass spectrometry (GCMS) [13]. The isotopic enrichment of leucine in VLDL<sub>1</sub>, VLDL<sub>2</sub> and IDL-apoB, and plasma  $\alpha$ KIC enrichment, was measured as described previously by GCMS [14]. Leucine enrichment in LDL<sub>2</sub> and LDL<sub>3</sub>-apoB was measured as the *N*-acetyl, n-propyl-ester derivative and analyzed by GC-combustion isotope ratio MS (Delta plus XP isotope ratio mass spectrometer, Thermo Scientific). Plasma <sup>2</sup>H<sub>2</sub>O enrichment was measured with a Gasbench II

inlet system and isotope ratio MS using platinum catalyst rods to liberate hydrogen gas. Isotopic enrichment was measured relative to laboratory standards previously calibrated against international standards Vienna Standard Mean Ocean Water and Standard Light Arctic Precipitation (International Atomic Energy Agency, Vienna, Austria). LPL and HL were measured in post-heparin plasma by the Confluolip Lipase test (Progen Biotechnik, Heidelberg). Plasma NEFA, total cholesterol, TAG, lipoprotein fraction TAG and cholesterol were measured by enzymatic assays using a Cobas MIRA (Roche, Welwyn Garden City, UK). Measurement of plasma adiponectin and leptin were by immunoassay (Millipore corporation, Billerica, MA, USA), apoB in lipoprotein fractions by an in-house ELISA and oxidised LDL by a commercially available ELISA (Biomedica, GmbH &Co Wien, Austria). Small dense (sd) LDL-cholesterol and plasma apolipoproteins CII, CIII and E were measured by precipitation methods (Randox Laboratories Ltd) on an iLab 650 (Werfen). *APO E* genotype was determined by quantitative polymerase chain reaction and Southern blotting, to exclude carriage of non-Apo E3 alleles.

#### *Data analysis*

Tracer enrichment of  $\alpha$ KIC, leucine, palmitate and glycerol was expressed as tracer/tracee ratio (TTR) corrected for baseline enrichment. Lipoprotein kinetics were calculated using compartment models as previously described [13,14]. These models and the calculation of the fatty acid contribution to VLDL-TAG PR are shown in the **Supplementary material**.

#### *Statistical methods*

Data from the end of each dietary period are expressed as arithmetic means for normally distributed variables, and  $\log_{10}$  transformed geometric means for non-normally distributed variables. The natural logarithm of each measurement, for the combined two sets (NAFLD and Controls) of the 2-period cross-over data, were analysed as dependent variables in a

general linear mixed model with the following fixed categorical, non-random, explanatory effects: treatment period, treatment (low and high sugar), liver fat level (NAFLD and Controls) and a treatment by liver fat level interaction, with subject as a model random effect using MIXED of SAS Version 9.2 (SAS Institute, Cary, NC, USA). Ratios and 95% confidence interval of measurements that were significantly different are shown in the Supplementary Material.

## **Results**

Twenty five men completed the study. The baseline characteristics of both groups, including age, body weight, BMI, waist circumference and biochemical measures were similar, except for plasma TAG which was 42% higher in men with NAFLD than Controls ( $p < 0.05$ ) (**Table 1**).

### *Dietary intake on high and low sugar diets*

Self-recorded dietary intakes and regular contact with participants indicated that dietary compliance was maintained. There was no difference in reported energy intake between diets (**Supplementary Table 1**). There were also no differences in energy intake, macronutrients or alcohol between NAFLD and Controls on either diets. The high sugar diet (26% total energy) produced a higher intake of total sugar and NMES in comparison to the baseline and low sugar diets (6% total energy) in both groups ( $p < 0.01$  for all comparisons). The high sugar diet was lower in starch ( $p < 0.001$ ) than the low sugar diet in both groups. Energy from dietary fat intake was significantly lower on the high sugar diet in Controls ( $p < 0.001$ ).

### *Change in body weight and plasma lipids*

Body weight was higher after the high versus low sugar diets in NAFLD ( $p < 0.001$ ) and Controls ( $p < 0.01$ ) (**Table 2**). However, there was no difference in body weight between NAFLD and the Controls after either diet. Plasma TAG was higher after the high sugar diet relative to the low sugar diet in both groups, but was not statistically significant. Plasma TAG was significantly higher in NAFLD relative to Controls after both the high ( $p < 0.01$ ) and low sugar diets ( $p < 0.01$ ), respectively.

### *Intra-abdominal and subcutaneous adipose tissue, and intra-hepatocellular lipid (IHCL) post-diet*

Post-dietary IHCL was significantly higher after the high versus the low sugar diet in both NAFLD (mean  $\pm$  SEM,  $24.2 \pm 6.8\%$  versus  $14.2 \pm 3.2\%$ ,  $p = 0.01$ ) and Controls ( $3.7 \pm 1.3\%$  versus  $1.4 \pm 0.3\%$  respectively,  $p < 0.01$ ) (**Fig 1A**). Men with NAFLD showed a greater increase in IHCL relative to Controls ( $p = 0.04$ ).

Visceral fat and subcutaneous adipose tissue mass was not different between groups, and was unaffected by diet in either group (**Supplementary Table 2**). Post-dietary IHCL was not associated with body weight, visceral fat, plasma TAG, or changes in these variables.

*Plasma lipoprotein concentrations and kinetics and post-heparin lipase activities*

**Between groups** Men with NAFLD were distinct from Controls by having higher concentrations of VLDL<sub>1</sub>-TAG and VLDL<sub>1</sub>-cholesterol, lower VLDL<sub>1</sub>-TAG fractional catabolic rate (FCR) and higher plasma apo-CIII on both the low sugar ( $p < 0.003$ ,  $p < 0.003$ ,  $p < 0.05$  and  $p < 0.01$  respectively) and high sugar diets ( $p < 0.02$ ,  $p < 0.02$ ,  $p < 0.05$  and  $p < 0.05$ ) (**Tables 3, 4 and Supplementary Table 4**). On the low sugar diet, NAFLD had higher total VLDL-TAG PR and VLDL<sub>1</sub>-TAG PR than Controls (both  $p < 0.05$ ).

**Between diets** In Controls, there was a higher PR of total VLDL-TAG (**Fig. 1B.**) and VLDL<sub>1</sub>-TAG (both  $p < 0.002$ ) and higher concentration of VLDL<sub>1</sub>-TAG and cholesterol (both  $p < 0.002$ ) after the high versus low sugar diet (**Tables 3 and 4**). In contrast, in NAFLD there was no significant difference in the PR of total VLDL-TAG or VLDL<sub>1</sub>-TAG, but a higher PR for VLDL<sub>2</sub>-TAG, IDL apoB, LDL<sub>2</sub> apoB and LDL<sub>3</sub> apoB (all  $p < 0.05$ ) (**Table 4**), and an increase in post-heparin hepatic lipase ( $p < 0.05$ ) (**Supplementary Table 4**) after the high versus low sugar diet. Although the TAG concentration in each individual fraction was not significantly different between diets in men with NAFLD, the sum of VLDL<sub>2</sub>, IDL, LDL<sub>2</sub> and LDL<sub>3</sub> TAG was higher ( $p < 0.05$ ) (data not shown). Oxidised LDL and sdLDL were

higher after the high sugar diet in men with NAFLD (both  $p < 0.05$ ) and Controls (both  $p < 0.02$ ) (**Table 3**).

*Palmitate kinetics and sources of fatty acids for VLDL<sub>1</sub>-TAG production*

**Between groups** Men with NAFLD were distinct from Controls by having a higher contribution of fatty acids to VLDL<sub>1</sub>-TAG PR from splanchnic fat on the high (both  $p < 0.04$ ) and low (both  $p < 0.01$ ) sugar diets (**Fig 1C**) (**Supplementary Table 3**). Men with NAFLD had a greater contribution of fatty acids from DNL to VLDL<sub>1</sub>-TAG PR than the Controls on the low sugar diet ( $p < 0.01$ ).

**Between diets** Plasma NEFA concentration, palmitate PR and MCR were all higher in men with NAFLD ( $p < 0.05$ ,  $p < 0.05$ ,  $p < 0.003$ ) after the high versus low sugar diet (**Supplementary Table 4**). There was a greater contribution of fatty acids from splanchnic fat to VLDL<sub>1</sub>-TAG PR ( $p < 0.02$ ) after the high sugar diet in Controls (**Fig 1C**) but although increased in NAFLD it did not achieve significance ( $p=0.075$ ). In NAFLD, there was a greater contribution of fatty acids from splanchnic fat to VLDL<sub>2</sub>-TAG PR ( $p < 0.02$ ) (**Fig 1D**) with no difference in Controls (between groups  $p < 0.002$ ). In Controls, there was a greater contribution of fatty acids from DNL ( $p < 0.002$ ) after the high sugar diet, but this was not evident in men with NAFLD. There was no significant difference in the contribution of systemic NEFA to VLDL<sub>1</sub> and VLDL<sub>2</sub>-TAG PR between diets in either group (**Fig 1C and D, Supplementary Table 3**).

## **Discussion**

This study provides new evidence for the effects of a high sugar diet in increasing liver fat and the PRs of the atherogenic lipoproteins (IDL and LDL) in men with NAFLD. A high sugar diet produced a greater contribution of fatty acids from splanchnic fat to the production of VLDL<sub>1</sub>-TAG, in Controls whereas in NAFLD there was a greater contribution of fatty acids from splanchnic fat to the production of smaller more atherogenic VLDL<sub>2</sub>-TAG.

The intake of sugar on the low sugar diet was close to the current recommendation for the intake of free sugars of no more than 5% total energy (NMES 6 ± 2% total energy or 586 kJ (140kcal) /day) [17, 18]. In contrast, the intake of sugar on the high sugar diet (NMES 26 ± 7% total energy) was five times greater than this recommendation (2,721kJ (650kcals)/day), but within the upper 2.5<sup>th</sup> percentile of a typical Western diet. Although there was a small difference in the intake of dietary fat between the iso-energetic diets, the overall metabolic response was consistent with the marked difference in intake of dietary sugar between the diets (20% of total energy).

It has been well documented that hyper-energetic, high sugar diets increase liver fat in healthy men [19], but there is less evidence that iso-energetic high sugar diets exert the same effect. A weight maintaining high fructose diet (25% total energy) has been reported to increase liver fat by 137% in healthy men [20] and an iso-energetic diet containing sucrose sweetened regular cola increased liver fat by 132% in overweight subjects [21]. To our knowledge the current study is the first to show: a) higher liver fat in men with NAFLD following a high versus low-sugar iso-energetic diet and b) the effect of the high sugar diet on liver fat is greater than in Controls.

There have been no studies to date on the effect of free sugar intake on VLDL kinetics in NAFLD. One study reported that VLDL-TAG PR in healthy subjects was higher after a 6-

day hypercaloric diet enriched with fructose as a liquid supplement (25% total energy) than after a 6-day, low-fructose diet [22]. VLDL-TAG PR was also higher after a 2-week high carbohydrate, low fat diet, compared to a 2-week iso-energetic, low carbohydrate high fat diet in healthy subjects [23]. In the present study, men with NAFLD on the low sugar diet, had a higher concentration and PR of large TAG-rich VLDL<sub>1</sub> than Controls with low liver fat, as shown previously in men on habitual diets [24]. In the Controls, the PR and plasma concentration of large TAG-rich VLDL<sub>1</sub> were higher on the high sugar diet compared to the low sugar diet. Moreover, the difference in PR of large TAG-rich VLDL<sub>1</sub> between groups was removed on the high sugar diet, as the values in Controls approached that of men with NAFLD, possibly because the Controls also gained liver fat. In men with NAFLD, although the PR of large TAG-rich VLDL<sub>1</sub> was higher on the high sugar diet, this was not significant; however the PR of the smaller more atherogenic VLDL<sub>2</sub> was significantly higher, suggesting an up-regulation of this pathway.

Men with NAFLD had a higher DNL relative to the Controls after both diets, consistent with the increased contribution of DNL to hepatic and lipoprotein fat in men with NAFLD [6, 25]. However, this finding was only significant on the low sugar diet, since the contribution of DNL to VLDL-TAG increased significantly in Controls but not NAFLD after the high sugar diet. DNL made only minor contributions (4-8%) to VLDL<sub>1</sub> and VLDL<sub>2</sub>-TAG production in both groups, and after both diets, as reported previously in healthy subjects [26]. DNL contributed approximately 12% of palmitate to VLDL-TAG in a previous study in NAFLD when measured over a comparable time period to the present study [6]. Previous studies have also shown that an 8 week diet with fructose-sweetened beverages, providing 25% of total energy, increased DNL, whereas glucose-sweetened beverages had no effect in healthy overweight participants [27]. Similarly, a 6-day high-fructose diet (25% total energy) increased DNL from 1.6 to 9.4% in VLDL-palmitate in healthy, normal weight men [28].

In the present study, there was no difference in the systemic contribution of fatty acids to VLDL<sub>1</sub>-TAG or VLDL<sub>2</sub>-TAG production between the diets, in both groups. This is perhaps surprising given the higher production and clearance rates of palmitate after the high sugar diet in NAFLD, which might be expected to result in increased delivery of NEFA to the liver. An increased contribution of fatty acids from splanchnic fat accounted for the majority of the higher VLDL<sub>1</sub>-TAG production on the high sugar diet in Controls and similarly in NAFLD the higher VLDL<sub>2</sub>-TAG, PR after the high sugar diet, was predominantly due to an increased contribution of fatty acids from splanchnic fat. This fat source consists of fatty acids from hepatic TAG storage pools and fatty acids from visceral adipose tissue, which drains directly into the liver via the portal vein. Hepatic TAG storage pools will expand in the fed, postprandial state, with an estimated 22% of dietary TAG being taken-up by the liver in chylomicron remnants [29], some of which will be stored and contribute to VLDL synthesis in the post-absorptive state [30]. The flux of NEFA from visceral adipose tissue has been estimated to be 17% of total NEFA delivery to the liver in obese men, but only 6% in lean men, and to correlate with visceral fat area by computer tomography [31]. However, since visceral fat was unaffected by the diets in the present study, this suggests that the relatively greater splanchnic contribution of NEFAs to VLDL-TAG production on the high sugar diet came from hepatic TAG storage pools. This would be consistent with the effect of dietary sucrose and fructose in augmenting postprandial lipaemia [32], and highlights the importance of postprandial TAG as a major source of lipid for the formation of an ALP via the accumulation of liver fat. It also suggests that the intra-hepatic fat pool is not inert, and there is an active process of turnover between post-prandial and fasting states.

Although the glycerol-3-phosphate pathway is the major pathway for TAG synthesis in the liver, an alternative pathway via monoacylglycerol acyltransferases (MGAT1-3) has been reported which is up-regulated in NAFLD (1). A possible source of monoacylglycerol for this

pathway is chylomicron remnants. Since an MGAT2-inhibitor has been shown to prevent carbohydrate induced fatty liver and suppress triglyceride synthesis and VLDL-secretion in mice fed a high sucrose very low fat diet (2), this pathway may contribute to the observed sugar induced VLDL2-elevation in the NAFLD subjects.

The ability of plasma lipoproteins to infiltrate the artery wall is a function of their particle size. Smaller VLDL<sub>2</sub> and IDL can cross the endothelial barrier and enter the arterial intima [33], while plasma IDL has been independently correlated with development of coronary atherosclerosis [34]. High sugar diets have been linked to the formation of oxidised LDL, and small, dense LDL (sdLDL) [27], a form of LDL with increased atherogenicity by virtue of its particle size, and susceptibility to undergo oxidative modification [35]. In our study, men with NAFLD had relatively higher production rates of IDL apoB, LDL<sub>2</sub> apoB and LDL<sub>3</sub> apoB after the high sugar diet, suggesting an increased flux of VLDL into IDL and LDL. These findings were further supported by a higher activity of hepatic lipase, an enzyme with a major role in the re-modelling of VLDL to IDL and LDL [39], and LDL into oxidatively susceptible sdLDL, after the high sugar diet.

In the present study, the two diets produced small, but remarkably similar effects on body weight in both groups, the high and low sugar diets being associated with a higher and lower weight ( $\pm 2\text{kg}$ ), respectively. Since this effects was the same in both groups, it suggests that the different responses to the diets between groups is unrelated to changes in body weight.

In conclusion, men with NAFLD were shown to be more sensitive to the effects of a high sugar diet in terms of their greater response in liver fat, and production of an ALP, relative to Controls. This suggests that a high sugar intake may have a role in the pathophysiology of NAFLD. Reducing intake of dietary sugar to levels recommended by dietary guidelines (5% total energy) may produce changes in plasma lipoproteins of men with fatty liver that are

beneficial to their cardio-metabolic health.

## Acknowledgments

We thank Jo Batt in the Faculty of Health and Medical Sciences, University of Surrey for technical assistance.

**Author contributions:** AMU, BG, JL and GF designed the study, FSM, JW, AM and XL performed the clinical studies, BG, JL, GF, CI and AA the dietary design and supervision, FSM, NJ, AM, XL, NA, MS and BF the laboratory work, supervised by AMU. RH and MW did the modelling, JB and ELT performed the MRI and MRS measurements, SJ performed the statistical analysis. BG and AMU were the lead writers. All authors were involved in drafting the article or revising it critically for important intellectual content and approved the final version. AMU is the guarantor of this work and, as such, had full access to all the data and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Financial support:** The work was supported by a UK government grant from the Biological Biotechnology Scientific Research Council (Grant no. BB/G009899/1); University of Surrey PhD scholarship for AM; Medical Research Council (body composition measurements) and infrastructure support from the National Institute of Health Research at the Cambridge Biomedical Research Centre.

**Disclosure:** JL has received financial support for other research studies from Sugar Nutrition UK and PepsiCo. All authors, including JL, declared there to be no duality of interest associated with this manuscript.

## Figure legends

**Figure 1:** In NAFLD and controls a) the effect of a low (grey bars) and high sugar diet (black bars) on percentage liver fat b) the effect of a low (grey bars) and high sugar diet (black bars) on VLDL TAG production rate c) the contribution of fatty acids from systemic (black bar), splanchnic (white bar) and DNL (grey bar) to VLDL<sub>1</sub> TAG production and d) the contribution of fatty acids from systemic (black bar), splanchnic (white bar) and DNL (grey bar) to VLDL<sub>2</sub> TAG production. Significantly different between diets, \* p<0.002; \*\*p<0.02; # p<0.05; † p=0.08. Significantly different between NAFLD and controls on low sugar diet, ‡ p<0.01. Significantly different between NAFLD and controls on high sugar diet, § p<0.04.

**Figure 2:** Schematic diagram showing the effect of a high sugar diet relative to a low sugar diet on lipoprotein metabolism in a) NAFLD and b) controls. Hatched arrows indicate significant changes. PR, production rate

## References

- [1] Stanhope KL, Schwarz JM, Havel PJ (2013) Adverse metabolic effects of dietary fructose: results from the recent epidemiological, clinical, and mechanistic studies. *Curr Opin Lipidol* 24:198-206.
- [2] Te Morenga LA, Howatson AJ, Jones RM, Mann J. (2014) Dietary sugars and cardiometabolic risk: systematic review and meta-analyses of randomized controlled trials of the effects on blood pressure and lipids. *Am J Clin Nutr* 100:65-79.
- [3] Marchesini G, Brizi M, Morselli-Labate AM et al (1999) Association of nonalcoholic fatty liver disease with insulin resistance. *Am J Med* 107:450-5.
- [4] Griffin BA, Freeman DJ, Tait GW, Thomson J, Caslake MJ, Packard CJ, Shepherd J (1994) Role of plasma triglyceride in the regulation of plasma low density lipoprotein (LDL) subfractions: relative contribution of small, dense LDL to coronary heart disease risk. *Atherosclerosis* 106:241-253.
- [5] Taskinen MR, Adiels M, Westerbacka J et al (2011) Dual metabolic defects are required to produce hypertriglyceridemia in obese subjects. *Arterioscler Thromb Vasc Biol* 31:2144-2150.
- [6] Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ (2005) Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest* 115:1343-1351.

- [7] Schwarz JM, Neese RA, Turner S, Dare D, Hellerstein MK (1995) Short-term alterations in carbohydrate energy intake in humans. Striking effects on hepatic glucose production, de novo lipogenesis, lipolysis, and whole-body fuel selection. *J Clin Invest* 96:2735-2743.
- [8] Jebb SA, Lovegrove JA, Griffin BA et al (2010) Effect of changing the amount and type of fat and carbohydrate on insulin sensitivity and cardiovascular risk: the RISCK (Reading, Imperial, Surrey, Cambridge, and Kings) trial. *Am J Clin Nutr* 92:748-758.
- [9] Szczepaniak LS, Nurenberg P, Leonard D et al (2005) Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of NAFLD in the general population. *Am J Physiol Endocrinol Metab* 288:E462-468.
- [10] National Diet and Nutrition Survey. Results from Year 1-4 (combined) of the Rolling Programme (2008/2009 - 2011/2012). (2014) Public Health England.
- [11] Department of Health. Dietary Sugars and Human Disease. Committee on Medical Aspects of Food Policy. Report on Health and Social Subjects No37. (1989) HMSO, London.
- [12] Kelly SA, Summerbell C, Rugg-Gunn AJ, Adamson A, Fletcher E, Moynihan PJ (2005) Comparison of methods to estimate non-milk extrinsic sugars and their application to sugars in the diet of young adolescents. *Br J Nutr* 94:114-124.

- [13] Sarac I, Backhouse K, Shojaee-Moradie F et al (2012) Gender differences in VLDL1 and VLDL2 triglyceride kinetics and fatty acid kinetics in obese postmenopausal women and obese men. *J Clin Endocrinol Metab* 97:2475-2481.
- [14] Brackenridge AL, Jackson N, Jefferson W et al (2009) Effects of rosiglitazone and pioglitazone on lipoprotein metabolism in patients with Type 2 diabetes and normal lipids. *Diabet Med* 26:532-539.
- [15] Thomas EL, Hamilton G, Patel N et al (2005) Hepatic triglyceride content and its relation to body adiposity: a magnetic resonance imaging and proton magnetic resonance spectroscopy study. *Gut* 54:122-127.
- [16] Watts GF, Mandalia S, Brunt JN, Slavin BM, Coltart DJ, Lewis B (1993) Independent associations between plasma lipoprotein subfraction levels and the course of coronary artery disease in the St. Thomas' Atherosclerosis Regression Study (STARS). *Metabolism* 42:1461-1467.
- [17] World Health Organisation's Draft Guidelines on sugar intake for adults and children (2014) [https://www.who.int/nutrition/sugars\\_public\\_consultation/en/](https://www.who.int/nutrition/sugars_public_consultation/en/) accessed 2 August 2015.
- [18] Scientific Advisory Committee on Nutrition: Draft report on carbohydrates and health (2014)[https://www.gov.uk/government/uploads/system/uploads/attachment\\_data/file/445503/SACN\\_Carbohydrates\\_and\\_Health.pdf](https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/445503/SACN_Carbohydrates_and_Health.pdf) accessed 5 August 2015.

- [19] Chung M, Ma J, Patel K, Berger S, Lau J, Lichtenstein AH (2014) Fructose, high-fructose corn syrup, sucrose, and nonalcoholic fatty liver disease or indexes of liver health: a systematic review and meta-analysis. Am J Clin Nutr 100:833-49
- [20] Schwarz JM, Noworolski SM, Wen MJ et al (2015) Effect of a High-Fructose Weight-Maintaining Diet on Lipogenesis and Liver Fat. J Clin Endocrinol Metab 100:2434-42
- [21] Maersk M, Belza A, Stødkilde-Jørgensen H et al (2012) Sucrose-sweetened beverages increase fat storage in the liver, muscle, and visceral fat depot: a 6-mo randomized intervention study. Am J Clin Nutr 95:283-9.
- [22] Theytaz F, Noguchi Y, Egli L et al (2012) Effects of supplementation with essential amino acids on intrahepatic lipid concentrations during fructose overfeeding in humans. Am J Clin Nutr 96:1008-16.
- [23] Mittendorfer B, Sidossis LS (2001) Mechanism for the increase in plasma triacylglycerol concentrations after consumption of short-term, high-carbohydrate diets. Am J Clin Nutr 73:892-899.
- [24] Adiels M, Taskinen MR, Packard C et al. (2006) Overproduction of large VLDL particles is driven by increased liver fat content in man. Diabetologia;49:755-765.
- [25] Lambert JE, Ramos-Roman MA, Browning JD, Parks EJ (2014) Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. Gastroenterology 146:726-35.

- [26] Timlin MT, Parks EJ (2005) Temporal pattern of de novo lipogenesis in the postprandial state in healthy men. *Am J Clin Nutr* 81:35-42.
- [27] Stanhope KL, Schwarz JM, Keim NL et al (2009) Consuming fructose-sweetened, not glucose-sweetened, beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. *J Clin Invest* 119:1322-1334.
- [28] Faeh D, Minehira K, Schwarz JM, Periasamy R, Park S, Tappy L (2005) Effect of fructose overfeeding and fish oil administration on hepatic de novo lipogenesis and insulin sensitivity in healthy men. *Diabetes* 54:1907-1913.
- [29] Bergman EN, Havel RJ, Wolfe BM, Bohmer T (1971) Quantitative studies of the metabolism of chylomicron triglycerides and cholesterol by liver and extrahepatic tissues of sheep and dogs. *J Clin Invest* 50:1831-1839.
- [30] Parks EJ, Krauss RM, Christiansen MP, Neese RA, Hellerstein MK (1999) Effects of a low-fat, high-carbohydrate diet on VLDL-triglyceride assembly, production, and clearance. *J Clin Invest*. 104:1087-96.
- [31] Nielsen S, Guo Z, Johnson CM, Hensrud DD, Jensen MD (2004) Splanchnic lipolysis in human obesity. *J Clin Invest* 113:1582-1588.

- [32] Abraha A, Humphreys SM, Clark ML, Matthews DR, Frayn KN (1998) Acute effect of fructose on postprandial lipaemia in diabetic and non-diabetic subjects. *Br J Nutr* 80:169-175.
- [33] NordesTAGaard BG, Wootton R, Lewis B (1995) Selective retention of VLDL, IDL, and LDL in the arterial intima of genetically hyperlipidemic rabbits in vivo. Molecular size as a determinant of fractional loss from the intima-inner media. *Arterioscler Thromb Vasc Biol* 15:534-542.
- [34] Hodis HN, Mack WJ, Azen SP et al (1994) Triglyceride- and cholesterol-rich lipoproteins have a differential effect on mild/moderate and severe lesion progression as assessed by quantitative coronary angiography in a controlled trial of lovastatin. *Circulation* 90:42-49.
- [35] de Graaf J, Hak-Lemmers HL, Hectors MP, Demacker PN, Hendriks JC, Stalenhoef AF (1991) Enhanced susceptibility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects. *Arterioscler Thromb*;11:298-306.
- [36] Zambon A, Bertocco S, Vitturi N, Polentarutti V, Vianello D, Crepaldi G (2003) Relevance of hepatic lipase to the metabolism of triacylglycerol-rich lipoproteins. *Biochem Soc Trans* 31:1070-1074.

**Table 1. Baseline characteristics.**

	<b>NAFLD (n=11)</b>	<b>Controls (n=14)</b>
Age y (range)	59 (49-64)	54(41-65)
Body weight kg	90.0±2.2	89.7±2.4
BMI kg/m <sup>2</sup>	28.9±0.3	28.4±0.5
Waist circumference cm	104±2	104±1
Liver fat %	17.2±2.7 <sup>b</sup>	2.5±0.3
Triacylglycerol mmol/l	1.89±0.27 <sup>a</sup>	1.33±0.23
Cholesterol mmol/l	5.91±0.25	5.51±0.28
HDL cholesterol mmol/l	1.22±0.08	1.24±0.08
Glucose mmol/l	5.73±0.11	5.46±0.12
Systolic BP mmHg	131±7	134±3
Diastolic BP mmHg	86±4.5	84±2.7

Values are means ± SEM. Significant difference between liver fat groups

<sup>a</sup>*P* <0.05, <sup>b</sup>*P*<0.001.

**Table 2. Effects of high and low sugar diets on anthropometrics, and plasma lipids**

	NAFLD (n=11)		Controls (n=14)	
	High sugar	Low sugar	High sugar	Low sugar
Body weight (kg)	89.7±2.5	87.7±2.4 <sup>c</sup>	88.9±2.8	86.7±2.9 <sup>b</sup>
BMI kg/m <sup>2</sup>	28.9±0.4	28.1±0.5	28.1±0.6	27.4±0.6
Body fat <sup>1</sup> %	27.6±0.8	26.3±0.8	24.4±0.7	23.6±0.9
Plasma TAG <sup>1</sup> mmol/l	2.05±0.24 <sup>a</sup>	1.77±0.22 <sup>a</sup>	1.33±0.15	1.13±0.08
Plasma Cholesterol mmol/l	5.59±0.33	5.24±0.30	5.10±0.25	4.82±0.26
Plasma LDL-C mmol/l	3.56±0.29	3.39±0.27	3.43±0.19	3.21±0.21
Plasma HDL-C mmol/l	1.18±0.09	1.13±0.07	1.16±0.07	1.13±0.08
Plasma Glucose mmol/l	5.44±0.09	5.30±0.09	5.08±0.14	5.11±0.08
Plasma Insulin mU/l	21.2±2.6	21.4±1.0	17.9±1.4	17.7±2.4
HOMA-IR	5.2±0.7	5.0±0.2	4.0±0.3	4.1±0.6

Values are arithmetic means ± SEMs unless stated otherwise. <sup>1</sup>Geometric mean ± SEM.

Significantly different between high and low liver fat groups <sup>a</sup>P <0.01. Significant difference between high and low sugar diets (post-diet) within liver fat groups <sup>b</sup>P <0.01, <sup>c</sup>P <0.001

**Table 3: Plasma lipoprotein fraction concentrations**

	NAFLD (n=11)		Controls (n=14)	
	High sugar	Low Sugar	High sugar	Low Sugar
VLDL <sub>1</sub> -TAG μmol/l	849±109 <sup>e</sup>	761±97 <sup>f</sup>	547±67	386±39 <sup>c</sup>
VLDL <sub>2</sub> -TAG μmol/l	147±21	110±10	104±11	104±14
IDL-TAG μmol/l	61±5	52±5	54±5	65±11
VLDL <sub>1</sub> -Chol μmol/l	345±76 <sup>e</sup>	283±49 <sup>f</sup>	207±26	127±14 <sup>c</sup>
VLDL <sub>2</sub> -Chol μmol/l	163±45	97±13	82±10	80±13
IDL-chol μmol/l	167±53	88±13 <sup>a</sup>	88±11	99±16
VLDL <sub>1</sub> -apoB mg/l	15.6±2.5	17.4±3.0	15.1±2.6	11.5±1.9
VLDL <sub>2</sub> -apoB mg/l	12.5±2.1	11.6±1.5	13.1±3.4	11.0±2.7
IDL-apoB mg/l	21.9±4.6	14.1±1.8 <sup>b</sup>	20.2±5.0	20.9±5.8
LDL <sub>2</sub> -TAG μmol/l	99±10	93±13	75±12	71±8
LDL <sub>3</sub> -TAG μmol/l	79±12	72±8	60±7	65±6
LDL <sub>2</sub> -chol μmol/l	1019±87	931±106	781±95	881±82
LDL <sub>3</sub> -chol μmol/l	1222±68	1252±60	1141±94	1172±45
LDL <sub>2</sub> -apoB mg/l	306±53	255±40	258±33	249±32
LDL <sub>3</sub> -apoB mg/l	567±92	574±98	570±48	459±53 <sup>a</sup>
Oxidised LDL μg/ml	5.22±0.0.36	4.07±0.55 <sup>a</sup>	4.98±0.55	3.36±0.33 <sup>b</sup>
Small dense LDL μmol/l	1459±210	1228±175 <sup>a</sup>	1043±112	848±78 <sup>b</sup>

Values are mean ± SEM. Ratios and 95% confidence interval of measurements that were

significantly different are shown in Supplement Table 5. Significantly different between

diets, <sup>a</sup>P<0.05; <sup>b</sup>P<0.02; <sup>c</sup>P<0.002. Significantly different between NAFLD and control group

on same diet, <sup>d</sup>P<0.05; <sup>e</sup>P<0.02; <sup>f</sup>p<0.003

**Table 4: Lipoprotein kinetic data**

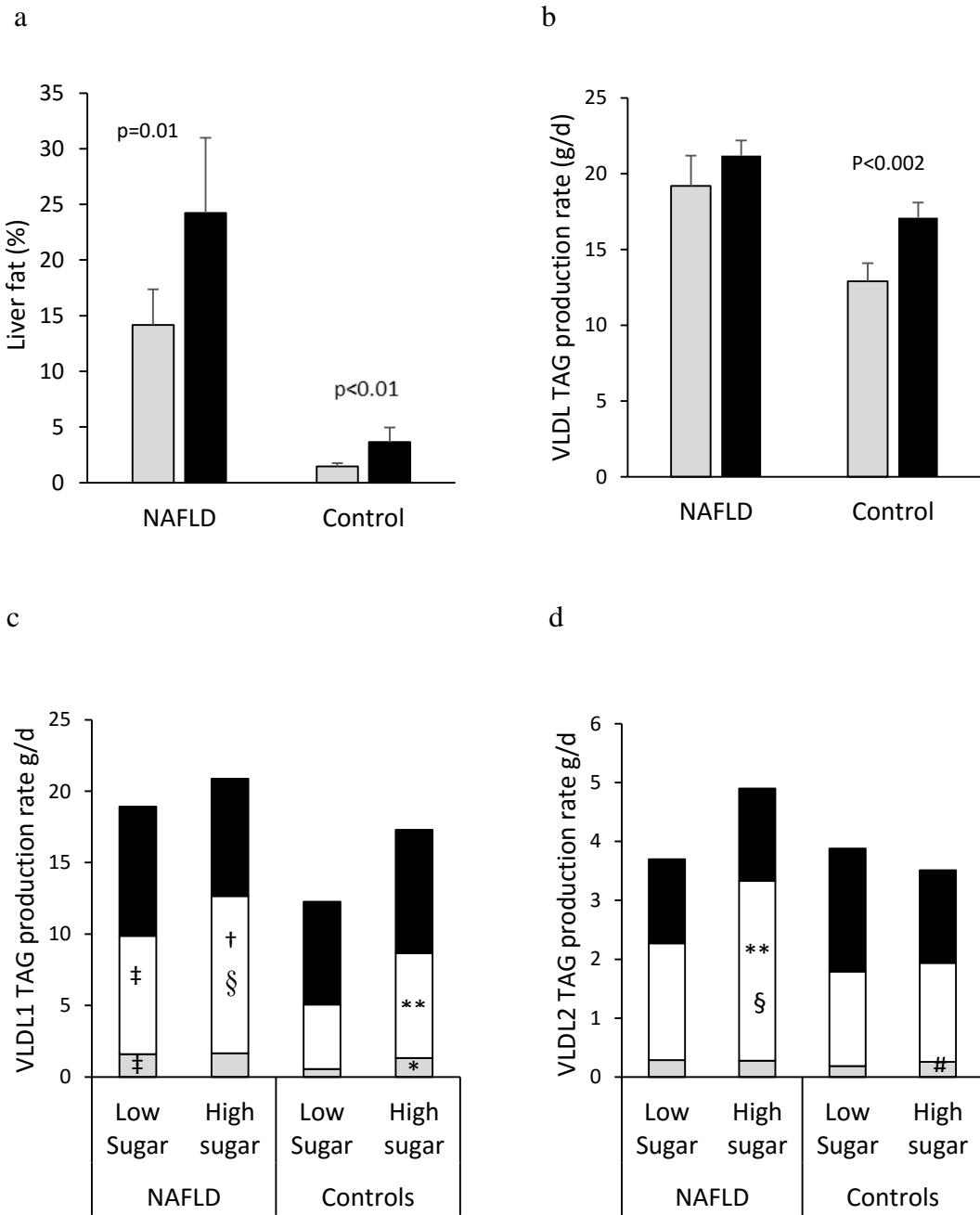
	NAFLD (n=11)		Controls (n=14)	
	High sugar	Low Sugar	High sugar	Low Sugar
VLDL <sub>1</sub> -TAG production rate g/d	20.9±2.1	18.9±2.1 <sup>d</sup>	16.6±1.4	12.4±1.1 <sup>c</sup>
VLDL <sub>1</sub> -TAG FCR pools/d	9.0±0.9 <sup>d</sup>	9.5±1.0 <sup>d</sup>	11.3±0.7	11.9±0.8
VLDL <sub>2</sub> -TAG production rate g/d	4.90±0.59	3.70±0.43 <sup>a</sup>	3.63±0.27	3.98±0.43
VLDL <sub>2</sub> -TAG FCR pools/d	11.5±1.1	12.2±1.3	13.1±1.0	14.3±0.9
VLDL <sub>1</sub> -apoB production rate mg/d	481±76	492±58	546±56	414±54
VLDL <sub>1</sub> -apoB FCR pools/d	9.0±1.0	10.8±2.2	14.7±2.7	13.4±2.4
VLDL <sub>2</sub> -apoB production rate mg/d	546±176	498±164	720±310	647±212
VLDL <sub>2</sub> -apoB FCR pools/d	12.5±2.8	12.8±2.4	13.6±1.9	14.9±1.6
Total VLDL-apoB production rate mg/d	577±71	566±69	680±73	524±58
IDL-apoB production rate mg/d	609±122	391±69 <sup>a</sup>	740±159	737±213
IDL-apoB FCR pools/d	9.5±1.9	8.7±0.9	12.2±1.1	12.1±1.2
LDL <sub>2</sub> -apoB production rate mg/d	1452±277	858±101 <sup>a</sup>	1075±109	1176±118
LDL <sub>2</sub> -apoB FCR pools/d	1.59±0.25	1.35±0.23	1.59±0.24	1.74±0.26
LDL <sub>3</sub> -apoB production rate mg/d	2069±388	942±278 <sup>a</sup>	1518±237	1374±273
LDL <sub>3</sub> -apoB FCR pools/d	1.01±0.15	0.46±0.09 <sup>c,d</sup>	0.86±0.12	1.06±0.24

Values are mean ± SEM. Ratios and 95% confidence interval of measurements that were

significantly different are shown in Supplement Table 6. Significantly different between diets, <sup>a</sup>P<0.05; <sup>b</sup>P<0.02; <sup>c</sup>P<0.002. Significantly different between NAFLD and control group on same diet, <sup>d</sup>P<0.05; <sup>e</sup>P=0.02; <sup>f</sup>P<0.003. In the High liver fat group, n=9 for the IDL and LDL2 kinetic data and n=8 for the LDL3 kinetic data due to insufficient data for the model fit.

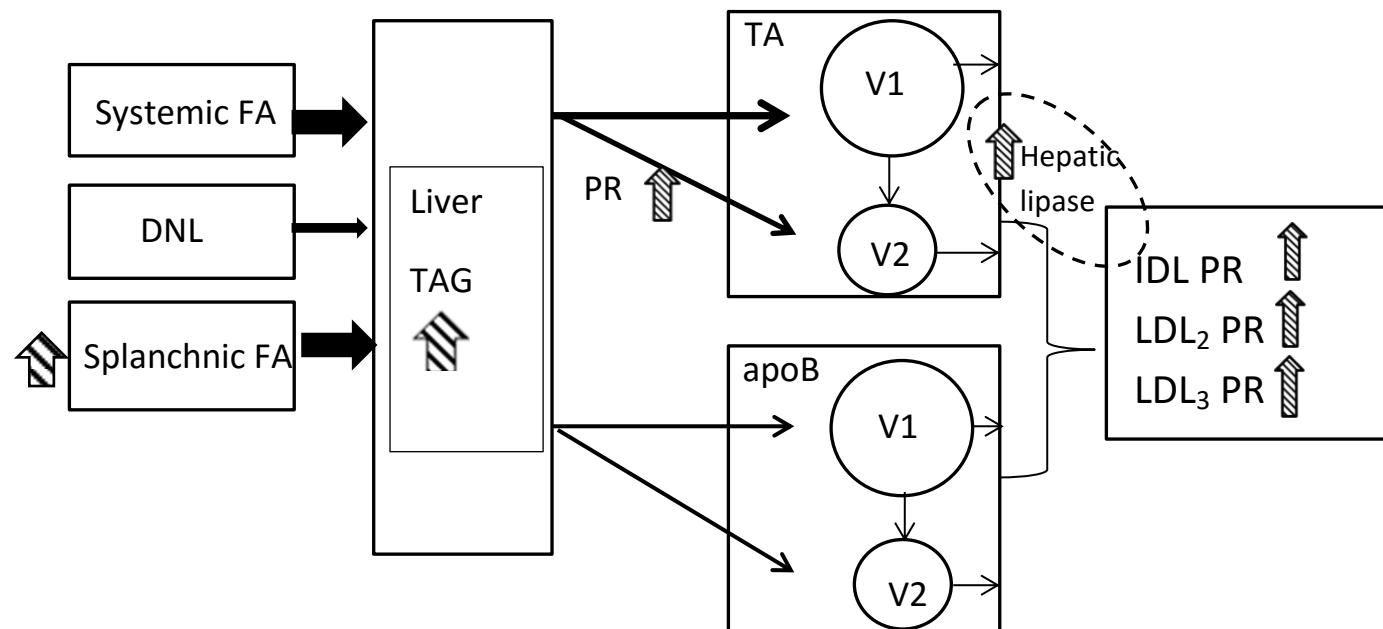


**Figure 1:** In NAFLD and controls a) the effect of a low (grey bars) and high sugar diet (black bars) on percentage liver fat b) the effect of a low (grey bars) and high sugar diet (black bars) on VLDL TAG production rate c) the contribution of fatty acids from systemic (black bar), splanchnic (white bar) and DNL (grey bar) to VLDL<sub>1</sub> TAG production and d) the contribution of fatty acids from systemic (black bar), splanchnic (white bar) and DNL (grey bar) to VLDL<sub>2</sub> TAG production. Significantly different between diets, \* p<0.002; \*\*p<0.02; # p<0.05; † p=0.08. Significantly different between NAFLD and controls on low sugar diet, ‡ p<0.01. Significantly different between NAFLD and controls on high sugar diet, § p<0.04.



**Figure 2** Schematic diagram showing the effect of a high sugar diet relative to a low sugar diet on lipoprotein metabolism in a) NAFLD and b) controls. Hatched arrows indicate significant changes. PR, production rate

A.



B.

