Effect of high- and low-fructose diets on intestinal fat production

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Glossary

BMI – body mass index

BP – blood pressure

Cedar – Centre for Diabetes and Endocrinology Research (RSCH)

CM – chylomicron

DNL – *de novo* lipogenesis

FAMEs – fatty acid methyl esters

%FM – percentage fat mass

FFM – Fat free mass

GCMS – Gas chromatography-mass spectrometry

GC-IRMS – Gas chromatography isotope ratio mass spectrometry

HDL – High-density lipoprotein

HTG – Hypertriglyceridemia

IEC – Ion exchange chromatography

LDL – Low-density lipoprotein

NEFAs – Non-esterified fatty acids

RIA – Radioimmunoassay

RSCH – Royal Surrey County Hospital

TRL – Triglyceride-rich lipoproteins

VLDL – Very low-density lipoprotein

WHR – Waist-to-hip ratio

1. Protocol Summary

TITLE:	Effect of high- and low-fructose diets on intestinal triglyceride production and de novo fatty acid synthesis
SHORT TITLE	Effect of high- and low-fructose diets on intestinal fat production
SPONSOR	University of Surrey
FUNDER REFERENCE	N/A
CLINICAL TRIALS GOV	[TBC]
DESIGN:	This will be a randomised crossover dietary intervention study, with all participants (n = 10; males; Caucasian; aged 18-50; BMI 25-32 kg/m^2) receiving diets high (30%) and low (<5%) in fructose, each for a 4-day period. Following completion of each diet, participants will attend fasted for a study day (12 hrs) at the Centre for Diabetes and Endocrinology Research (Cedar) at Royal Surrey County Hospital (RSCH). Volunteers will have anthropometric (height; weight; waist-to-hip ratio; fat free mass) and blood pressure measurements taken and give a fasting blood sample. They will then consume hourly meals to establish a steady post-prandial state and have blood samples taken at regular intervals. A bolus of $^2H_5$-glycerol isotopic tracer will be given 4 hours into the study to measure the production rate of triglyceride (TG) in plasma chylomicron and VLDL lipoproteins. Water enriched with ^2H_2O will also be given exclusively from the evening prior to the study day, until the end of the feeding protocol, to calculate *de novo* lipogenesis (DNL). A pilot study (n ≤ 3) will also be conducted prior to the start of the main study described above, to optimise the feeding protocol used. Participants will attend a single study day at the Cedar centre and will not receive the $^2H_5$-glycerol tracer or dietary intervention.
OVERALL AIM:	To determine the different effects of high and low fructose diets on intestinal chylomicron TG production and DNL.
PRIMARY OBJECTIVES	The effect of high and low fructose diets on chylomicron TG production.
SECONDARY OBJECTIVES	N/A
Target Accrual	Pilot study n ≤ 3, Main study n = 10
Inclusion Criteria	- Male - Caucasian - A Body Mass Index (BMI) of 25-32 kg/m^2. - Aged 18-50 (main study) or 18-65 years old (pilot study) at time of recruitment. - Less than 3 sessions of aerobic exercise (>30 min) per week.
Exclusion Criteria	- Type 1 or Type 2 diabetes. - Subjects diagnosed with endocrinological or cardiovascular disease, renal impairment or gastrointestinal and liver diseases (except fatty liver disease). - A fasting blood glucose >7 mmol/l or fasting triglyceride

	>4mmol/l - An alcohol intake exceeding 32g/day (4 units) or history of drug abuse. - On a weight-reducing diet. - Smokers. - Any relevant food allergies or intolerances e.g. fructose intolerance, allergy to sweetener(s) used in drinks. - Prior/present history of eating disorders (anorexia, bulimia nervosa) - Prior/present history of prolonged nausea or vomiting - History of stomach/gastric surgery - Those taking a fibrate drug - Those taking the drug Metformin
Number of Sites	1 NHS site (Cedar centre)
Duration of Recruitment	6 months
Duration of Patient Follow-up	N/A
Definition of End of Study	When all participants have completed both diets and study days and all laboratory analyses have been completed.

2. Background

Literature Review and Rationale for Study

Fructose is one of the three main dietary monosaccharides, with common sources including fruit, honey and table sugar (sucrose). Fructose has only a minimal effect on insulin secretion, compared to glucose, and thus a lower glycaemic index. It is also the sweetest naturally-occurring carbohydrate (1) and has been considered a suitable replacement for sucrose in cases of obesity and type 2 diabetes.

However, research has linked fructose consumption in humans to several adverse effects, including increased hepatic fat deposition (2), decreased hepatic insulin sensitivity (2-3), an increase in small dense LDL (4), as well as increased fasting (5-6) and post-prandial triglyceride (7-9) concentrations (hypertriglyceridaemia – HTG).

Both fasting and post-prandial HTG are well-recognised risk factors for cardiovascular disease (10), as well as being predictive of cardiovascular outcomes involving hospitalisation or mortality (11). As CVD is the leading cause of death globally (30%), accounting for an estimated 17.3 million deaths in 2008 (12), the impact of fructose on triglyceride (TG) metabolism has been the subject of much research.

Hypertriglyceridaemia is the result of excess triglyceride-rich lipoproteins (TRL) in the circulation, comprising very low-density lipoproteins (VLDL) and chylomicrons (CM). These TG carrying particles are synthesised by the liver (VLDL) and intestine (CM) respectively to distribute dietary fat to peripheral tissues, such as skeletal muscle and adipose tissue, for either energy generation or storage. Both VLDL and CM are hydrolysed by the endothelial enzyme lipoprotein lipase (LPL) in peripheral tissues, thus releasing fatty acids and leading to smaller, denser, lipid-poor 'remnant' particles, which are cleared from the circulation by a number of receptor-mediated pathways (13).

Post-prandial HTG may therefore result from either an increase in the production of VLDL and/or CM particles, an increased particle TG content, or a reduced catabolism and clearance of remnant

particles from the circulation. Most studies have investigated VLDL contribution to HTG. However, one study using a hamster model demonstrated that chronic fructose feeding for 3 weeks increased the production of intestinal lipoproteins, as well as de novo lipogenesis (DNL), in both the fasting and fed states (14). In humans, it has been shown that men with metabolic syndrome exhibit a greater post-prandial production of CM TG than lean controls, in response to identical meals, with no difference in the rate of clearance (15).

Consumption of a high-fructose (30% of energy) diet for 4 days in healthy non-obese males was shown to increase TRL TG in both the fasted state and following an oral fructose challenge, in comparison to a low-fructose diet [<5%] (7). In addition, almost twice the amount of fasting chylomicron-like particles (inferred from the apoB48 concentration) were observed in subjects after consuming the high-fructose diet, although the contribution of intestinal DNL to the increased TRL TG could not be estimated in this study.

In an acute study comparing the effects of an infusion of intralipid and fructose versus intralipid and saline, total plasma TG and the production of chylomicron particles increased due to fructose (16). However, the specific effect on CM TG was again not assessed.

The current study will aim to further investigate the specific mechanism(s) of fructose-induced HTG by determining the effect of a high-fructose (30% of energy) diet, compared to a low-fructose (<5% of energy) one, on intestinal CM TG production and DNL in healthy overweight male volunteers, using a 'steady-state' continuous feeding protocol and stable isotope tracers. It is anticipated that the high-fructose diet will lead to an increase in these two parameters, demonstrating a potential role of the intestine in fructose-induced HTG.

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3. Study Objectives

Primary

To compare the effects of high and low fructose diets on the production of chylomicron TG.

4. Participant Population

Inclusion criteria

- Male
- Caucasian
- A Body Mass Index (BMI) of 25-32 kg/m2.
- Aged 18-50 (main study) or 18-65 years old (pilot study) at time of recruitment.
- Less than 3 sessions of aerobic exercise (>30 min) per week.

Exclusion criteria

- An inappropriate BMI or age.

- Non-Caucasian
- Type 1 or Type 2 diabetes.
- Subjects diagnosed with endocrinological or cardiovascular disease, renal impairment or gastrointestinal and liver diseases (except fatty liver disease).
- A fasting blood glucose >7 mmol/l or fasting triglyceride >4mmol/l
- An alcohol intake exceeding 32g/day (4 units) or history of drug abuse.
- On a weight-reducing diet.
- Smokers.
- Any relevant food allergies or intolerances e.g. fructose intolerance, allergy to sweetener(s) used in drinks.
- Prior/present history of eating disorders (anorexia, bulimia nervosa)
- Prior/present history of prolonged nausea or vomiting
- History of stomach/gastric surgery
- Those taking a fibrate drug
- Those taking the drug Metformin

5. Participant Recruitment

Participants will be recruited primarily through the University of Surrey. The PhD student (Mr Simon Steenson) will be responsible for recruitment, under the supervision of senior members of the research team with experience in recruitment. Participants will also be sought from the Guildford area via the use of advertisements placed in shops and other public places. An internal database compiled by the research group, containing respondents to adverts from previous studies, may also be used. All people in the database have agreed to be contacted regarding future studies and would undergo the same process of informed consent and screening as for all participants.

6. Dietary Intervention

Participants will be given two different controlled diets, each for 4 days, composed of common foods as well as drinks containing either fructose (high-fructose diet) or a calorie-free sweetener (low-fructose diet). The energy content of both diets will be matched as closely as possible to each participant's calculated energy requirements (Henry equation) and will be supplied to participants for them to consume at home.

7. Study Design

Screening visit (pilot and main study)

Upon making initial contact, potential participants will be given verbal/written information about the study via telephone or email, and they will be asked for their contact details and some basic health questions to assess their suitability for the study according to the inclusion/exclusion criteria. This will ensure that only suitable volunteers are invited for screening. A participant Information Sheet will be sent to potential participants via email or post and they will be given a minimum of 24 hours to consider whether they wish to take part.

Those who are willing to participate will be requested to attend the Centre for Diabetes and Endocrinology Research (Cedar) at Royal Surrey County Hospital (RSCH) following an overnight fast.

Volunteers will be given further verbal and written information about the study, including the number of visits and the procedures involved, and be fully informed of their rights and responsibilities while participating in the study. A set of health questions will be asked, similar to those answered at initial contact, as well as the opportunity for participants to request further information.

Those wishing to participate will be required to sign and date an informed consent form (the same for the pilot and main study) and will be given a copy to keep. The following will be measured or recorded at the screening visit: health questionnaire (inclusion/exclusion criteria information, current medication); diastolic and systolic blood pressure; age; height; weight; haematocrit; blood triglyceride, glucose and total cholesterol. If the participant fulfils all of the inclusion criteria and none of the exclusion criteria then they will be accepted into the study. Eligible participants from the screening process will be invited to attend the Cedar for either the pilot or main study. Participants from the pilot study may also be recruited for the main study, although additional informed consent will be obtained.

Pilot Study

Baseline Blood Sample (Cedar centre – 30 min)

Participants will be asked to attend the Cedar centre (non-fasted) to give a baseline blood sample (30 min) on the day prior to attending for a 'study day', which is necessary to determine their background level of naturally-occurring $^{2}H_2O$ water. Participants will be provided with a standardised meal to eat at 7pm that evening, as well as two small bottles containing a 'loading dose' of $^{2}H_2O$ (3g/kg) - one to be consumed after the evening meal and the other at 10pm. They will be provided with additional $^{2}H_2O$ water (4.5g $^{2}H_2O$/litre) to drink exclusively until the end of the following day (study day), in order to maintain $^{2}H_2O$ enrichment in the blood throughout the study day protocol.

Study Day (Cedar centre – 12 hours)

Subjects will attend the Cedar Centre the next day at 7.30am, having fasted overnight. They will have their height (cm), weight (kg), BMI, %FM and FFM, BP and WHR measured. An intravenous cannula will be inserted into an antecubital vein to allow for blood sampling. A fasted blood sample will be taken, followed by consumption of a small meal, either solid or liquid, with a composition similar to the intended dietary intervention of the main study. Subsequent meals will be consumed on an hourly basis to establish a 'steady post-prandial' fed state (11 hour protocol). The total energy content of all meals will be matched as closely as possible to each participant's calculated energy requirements. Blood samples will be taken at regular intervals throughout the day. At the end of the feeding protocol, participants will be offered something to eat (e.g. sandwich, drink and snack) and be free to go home.

Following this visit, blood samples will be analysed and the results used to optimise the feeding protocol, leading to recruitment for the main study.

Main Study

Dietary Intervention (4 days)

Following screening and recruitment into the study, participants will be supplied with all of the food and drinks for either the high (30% of energy) or low (<5% of energy) fructose diet. Diets will be matched to each participant's calculated energy requirements, with the drinks containing either fructose (high fructose diet) or a calorie-free sweetener (low-fructose diet).

Baseline Blood Sample (Cedar centre – 30 min)

Participants will be asked to attend the Cedar centre (non-fasted) to give a baseline blood sample (30 min) on the final day of each diet (afternoon), prior to attending for each study day. This is to allow measurement of the background level of $^{2}H_2O$ in the participant's blood, as in the pilot study, and the protocol for this visit will be the same.

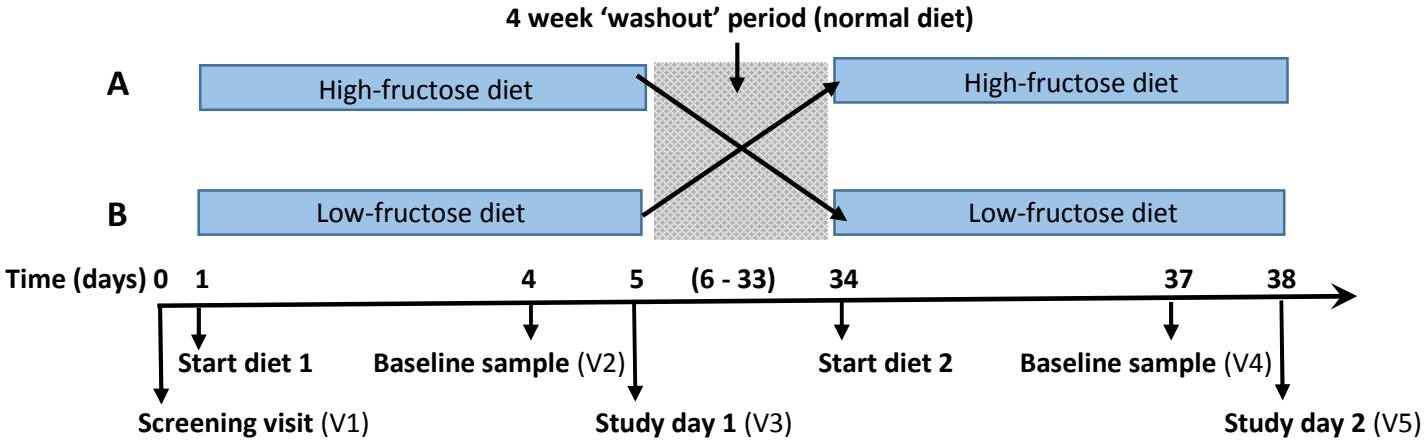
Study Days (Cedar centre- 12 hours, twice during study)

As for the pilot study, participants will attend the Cedar Centre the next day at 7.30am, having fasted overnight. They will have their height (cm), weight (kg), BMI, %FM and FFM, BP and WHR measured. An intravenous cannula will be inserted into an antecubital vein to allow for blood sampling. A fasted blood sample will be taken, followed by consumption of hourly meals, with a composition similar to the diet received during the previous 4 days.

Subsequent meals will be consumed on an hourly basis (11 hours in total) during the study to establish a 'steady post-prandial' fed state. The total energy content of all meals will be matched as closely as possible to each participant's calculated energy requirements. Blood samples will be taken at regular intervals throughout the day. Four hours after the start of the feeding protocol, a bolus of intravenous $^{2}H_5$-glycerol will be administered via a separate cannula, inserted in the opposite arm, to trace chylomicron TG production in the intestine and also VLDL production in the liver.

Washout period and crossover to second dietary intervention

Following the study day, participants will undergo a 4-week washout period, consuming their usual diet, and then crossover to receive the second dietary intervention (high or low fructose), depending on which was not previously consumed. Participants will again consume the diet for 4 days and attend for a baseline blood sample and study day at the Cedar centre as described above. The diagram below illustrates the design of the main study, including the timing and number of visits required.



8. Data Analysis

Laboratory Analyses

All participant samples will be analysed in the Diabetes and Metabolic Medicine research laboratories (Leggett building, University of Surrey) according to established protocols.

Separation of VLDL and chylomicron triglyceride rich lipoproteins by immunoaffinity method

The TRL fraction, containing VLDL and chylomicrons, will be separated from plasma samples (3 ml) at each time point using ultracentrifugation (Beckman Coulter, Optima LE-80K, UK). Samples will then be made up to 2 ml with saline. Subsequently, 1 ml will be used for the determination of apoB48 (chylomicrons) and apoB100 (VLDL) lipoprotein concentrations via an ELISA method. The other 1 ml will undergo an immunoaffinity purification method to separate the apoB100 and apoB48 fractions using a sequential binding method involving 3 distinct monoclonal antibodies, bound to protein G sepharose 4. Before separation, the Protein G sepharose is made into a slurry and washed with H2O, 1mM HCl and Na2HPO4 before being mixed with the 3 antibodies to apoB100 (4G3, 5E11 and Bsol 16). Antibody binding is verified using bovine serum albumin, before samples are incubated sequentially with each of the three Protein G-antibody complexes overnight. The resulting 3 bound (apoB100) layers and 3 unbound (apoB48) fractions are each pooled together, yielding one total bound and unbound sample for each time point.

TG extraction, purification and hydrolysis

TG extraction will be achieved through the use of chloroform:methanol (2:1 v/v) and 0.99% KCl, combined with subsequent spin cycles. The resulting samples will have 100 µl of ethanol added and be dried down under oxygen-free nitrogen overnight, after which 100 µl of chloroform will be added. Samples will then be loaded onto plates for thin layer chromatography (TLC) to yield TG bands, which will be scraped off and hydrolysed in 1 ml toluene and 2 ml 3% HCl (50^{o}C overnight) with an internal standard added (heptadecanoic acid and ^{13}C-glycerol). After cooling, 2 ml 5% NaCl and 3 ml hexane will be added to each sample. Vials will be refrigerated at 4^{o}C for at least 30 minutes and centrifuged for 10 minutes at 2500 rpm, thus yielding glycerol in the aqueous layer and fatty acid methyl esters (FAMEs) in the solvent layer. FAMEs will then be ready for quantification using GCMS.

The aqueous layer samples, containing glycerol, will be purified via ion exchange chromatography (IEC), freeze-dried and then derivatised in the presence of 100 µl of ethyl acetate before quantification using GCMS. Samples taken for the determination of plasma glycerol will also undergo the same process of IEC, freeze-drying and derivatisation before analysis by GC-IRMS.

Measurement of 2H2O by GC-IRMS

The enrichment of $^{2}H_{2}O$ in plasma samples will be analysed using an isotope ratio mass spectrometer with a Gasbench II inlet system. Sample tubes will be capped, flushed with the equilibration gas (5% 2H2 in helium at 100ml/min) and incubated for a minimum of 40 minutes at 22.5 ^{o}C, before a platinum catalyst rod is used to measure isotopic enrichment relative to laboratory standards (calibrated against Standard Mean Ocean Water and Standard Light Arctic Precipitation [International Atomic Agency, Vienna]).

Data analysis

The production rate of chylomicron and VLDL TG, as well as intestinal DNL (CM palmitate), will be calculated by assessing the levels of tracer enrichment at baseline, beginning of the metabolic study days (fasted) and post-prandially during the feeding protocol (steady fed state).

Tracer enrichment of palmitate and glycerol will be expressed as tracer/tracee ratio (TTR) corrected for baseline enrichment. VLDL and CM-TG fractional catabolic rate (FCR) will be calculated using a compartment model using the SAAM II program (SAAM Institute, Seattle, WA). The fractional contribution of DNL to VLDL and CM-TG synthesis will be calculated from the deuterium enrichment in VLDL and CM-TG palmitate and in plasma water. The deuterium enrichment that would have been obtained if endogenous synthesis were the only source of plasma TG-fatty acids will be calculated from plasma water enrichment. Comparison of the actual enrichments observed with these theoretical values provides a measure of the percent contribution, expressed as fractional synthesis of plasma TG. The contribution of DNL to VLDL and CM-TG production rate will be calculated by multiplying DNL fractional synthesis rate by the respective TG production rates.

Other measurements

The following parameters will be assessed using enzymatic methods by a Cobas MIRA analyser, according to established protocols: total plasma TG, glucose, cholesterol, NEFAs and HDL-cholesterol, as well as CM and VLDL cholesterol. Plasma insulin will be assessed by RIA.

Statistical Considerations

Determination of statistical power

Data from a previous study by our research group found the chylomicron TG production rate in 7 healthy individuals to be 11.82 ± 5.26 g/day (mean ± SD). For the current study we plan to recruit 12 participants in order to give a sample size of 10 subjects, assuming a dropout of 2 subjects. Using a two-sided t-test with a power of 80% and $\alpha = <0.05$, this would yield a smallest detectable mean difference of 7.439 g/day for chylomicron TG production rate, the primary outcome measure.

Data Management

All personal identifiable data will be either kept on secure University servers or password protected machines (electronic data), or in locked metal filing cabinets accessible only to members of the research team (hard copy data), located within a building requiring card access (Leggett Building). Screening sheets and consent forms, will be coded with a participant identification number. These documents will be the only connection to the participants' personal details. All other study documents, blood samples and data will use the trial ID number for identification purposes.

Only data with the study ID number will leave the university's secure network. No personal identifiable data will be transferred electronically using electronic storage media, email or computer networks.

9. Adverse Events

The potential risk of adverse events (AE) for this study is anticipated to be low. Some participants may experience a slight and transient dizziness due to the $^{2}H_2O$-enriched water given during the protocol, although instances of this are rare. It is also possible that some participants may not tolerate the fructose in the diet, or given as part of the liquid meals, although they will be asked about history of fructose intolerance during the screening process. A trained medical professional will be available if required to assist in dealing with these problems.

Reporting Procedures

While the incidence of AEs in this study is anticipated to be very minimal, all AEs shall be recorded using a standardised form. In the unlikely event of more serious AEs, other than those already mentioned, appropriate medical attention will be sought for the participant and their GP will be informed.

10. Withdrawal of a participant

Patients can withdraw from the study at any stage without giving a reason. All data collected up to the point of withdrawal will be included in the trial analysis. However, if the participant withdraws consent for their data to be used the data will be destroyed immediately.

11. Study closure

End of Study
Upon the end of the study a "declaration of end of trial" form will be submitted to REC, as required. The study will be considered complete when all participants have finished both dietary interventions and study days and all laboratory and statistical analyses have been carried out.

Archiving study documents
Following the end of the study all information will be stored securely for at least 10 years in accordance with the University of Surrey's policy.

12. Sponsorship

The current study is sponsored by the University of Surrey and funded jointly by the University and the BBSRC through the Food Security Doctoral Training Partnership (50:50).

13. Indemnity

Any harm to participants arising from the conduct of the research, is covered by the NHS Indemnity scheme or through professional indemnity. The research doctor collaborating on the study, Dr Martin Whyte, has suitable professional liability cover as a practising clinician.

The University of Surrey has suitable indemnity for public and employer liability for research purposes, which will provide cover for harm to participants arising from the design and management of the research.

14. Publication

The results of this study will be published in a peer-reviewed journal and presented at national/international conferences.