Protocol

Study title: Revealing the mechanisms by which milk sugars exaggerate postprandial lipaemia

Short title: Lactose & Lipids

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Sponsor: University of Bath

Funder: British Heart Foundation

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1. SYNPOSIS

Study Title	Revealing the mechanisms by which milk sugars exaggerate postprandial lipaemia
Short Title	Lactose & Lipids
Trial Design	Randomised Controlled, Crossover Trial in men and women
Trial Participants	Men and women aged 18-50 years and premenopausal (for women)
Intervention	Acute ingestion of three test drinks: Maltodextrin (Control) Lactose (Intervention) Sucrose (Active comparator)
Planned Sample Size	24 (12 men and 12 women)
Primary Objective	To determine to what extent ingestion of lactose exaggerates postprandial triglyceridaemia in men and women.
Secondary Objectives	To determine the effect of lactose ingestion on hepatic fatty acid synthesis (*de novo* lipogenesis). To determine the degree to which lactose ingestion alters dietary fat oxidation.
Primary Outcomes	The effect of lactose *versus* maltodextrin co-ingestion on postprandial incremental area under the curve (iAUC) for plasma triglyceride concentrations (6-hour postprandial period).
Secondary Outcomes	The effect of treatment (lactose versus maltodextrin co-ingestion on: 1) Postprandial de novo lipogenesis (DNL) 2) Dietary fat oxidation
Tertiary Outcomes	Treatment (lactose *versus* fructose *versus* maltodextrin) by sex (male *versus* female) interaction effect on: 1) Postprandial incremental area under the curve (iAUC) for plasma triglyceride concentrations (6-hour postprandial period). 2) Postprandial de novo lipogenesis (DNL) 3) Dietary fat oxidation 4) Postprandial iAUC for plasma insulin concentrations 5) Postprandial iAUC for plasma glucose concentrations 6) Postprandial iAUC for plasma galactose concentrations 7) Postprandial iAUC for plasma fructose concentrations 8) Postprandial iAUC for plasma VLDL-rich triglyceride [Svedberg flotation rate (S_f): 20-400] concentrations 9) Postprandial iAUC for plasma chylomicron-rich triglyceride (S_f: >400) concentrations 10) Postprandial iAUC for plasma lactate concentrations 11) Postprandial iAUC for plasma non-esterified fatty acid concentrations 12) Postprandial iAUC for plasma glycerol concentrations 13) Postprandial iAUC for plasma beta-hydroxybutyrate concentrations 14) Postprandial iAUC for plasma uric acid concentrations
Funder	British Heart Foundation (PG/19/43/34432)
Sponsor	The University of Bath
Principal Investigator	Dr Javier Gonzalez
Chief Investigator	Dr Javier Gonzalez

2. BACKGROUND AND RATIONALE

2.1. Executive Summary

Elevated postprandial triglycerides play a causal role in cardiovascular disease (CVD). Restricting sugar intake can reduce circulating triglycerides and nutrition guidelines recommend restricting free sugar intakes for health. Importantly, milk sugars are currently omitted from this restriction simply due to an insufficient evidence-base to draw firm conclusions. Our preliminary data demonstrate that galactose (the unique component of the milk sugar lactose) increases postprandial triglyceride concentrations in lean men, produces a similar metabolic milieu to fructose ingestion, and stimulates triglyceride accumulation in hepatocytes. If galactose exaggerates postprandial triglyceridaemia via common mechanisms to fructose, then this has important health and policy implications due to the potential additive effect of consuming milk products containing free sugars. This project will achieve the following objectives: 1) generate data on the role of lactose in postprandial lipaemia in men and women; 2) establish if hepatic fatty acid synthesis, VLDL availability, and/or changes in handling of dietary fat in the postprandial state are the underlying mechanism(s) by which lactose exaggerates postprandial lipaemia. These data will provide key evidence required to justify longer-term studies, used to refine nutrition guidelines on sugars and CVD. This is timely, as inclusion of milk-based drinks in the Soft Drinks Industry Levy is currently being considered.

2.2 Relevant Literature and Pilot Data

Cardiovascular disease (CVD) is a global health burden and postprandial triglycerides play a major causal role in CVD risk. Cardiovascular disease is the leading cause of death worldwide, responsible for ~17 million of the ~54 million deaths in 2013[1]. The total cost of CVD is estimated to rise from $863 billion in 2010 to $1044 billion by 2030[2]. With such a huge social and economic burden, prevention is understandably a global priority. Mendelian randomisation suggests a **causal role** of plasma triglyceride concentrations in coronary heart disease[3]. Furthermore, it is important to consider *postprandial* triglyceride concentrations (as opposed to post-absorptive) for at least two main reasons. First, in contrast to the fasted state, the fed state captures the total amount of atherogenic lipoproteins in plasma (from both hepatic and intestinal origin); and second, the postprandial state predominates most of the 24-h cycle for the majority of populations in developed countries, thereby better reflecting habitual exposure to atherogenic lipoproteins directly involved in CVD[4]. Individuals with postprandial plasma triglyceride concentrations above 4 $mmol \times L^{-1}$ have a >5-fold increased CVD mortality compared to those with triglyceride concentrations below 1 $mmol \times L^{-1}$[5]. Therefore, there is an urgent need to uncover strategies that in reduce postprandial plasma triglyceride concentrations and thus, CVD risk.

More evidence on the health effects of milk sugars in humans is essential to inform nutrition guidelines. Nutrition is a major determinant of plasma triglycerides and CVD risk. Accordingly, public health authorities (e.g. British Heart Foundation, the Scientific Advisory Committee on Nutrition and the World Health Organisation) provide dietary recommendations for health[6; 7; 8], which consistently advise reducing intakes of free sugars. Indeed, there are metabolic health consequences to the overconsumption of sugars such as fructose; shown to exaggerate postprandial lipaemia and hepatic triglyceride synthesis (i.e. *de novo* lipogenesis; DNL), whilst suppressing fat oxidation[9; 10; 11]. Importantly, milk sugars are specifically omitted from the current definition of free sugars and thus no upper limit of sugar intake from dairy is provided[7]. This decision is based on "*no reported evidence of adverse effects of consumption of intrinsic sugars and sugars naturally present in milk*"[7]. Whilst this is an appropriate recommendation based on the extent of current evidence available, the statement is based on lack of evidence of an effect, rather than evidence for a lack of an effect. Moreover, sugar intake from dairy products is not negligible, with dairy products contributing more than 20% of total sugar intake in many countries[12], and more than 95% of the UK population report regularly consuming dairy products[13]. Thus, a better understanding of the health impact of milk sugars should be a priority. The only randomised, controlled study in humans to measure plasma triglycerides in response to galactose supplementation did show that *fasting* plasma triglyceride concentrations were increased by ~30% with 4 days of galactose supplementation compared to glucose supplementation[14]. In the absence of relevant human data, the best available evidence to date, for the impact of milk sugars on CVD comes from studies on non-human primates. Baboons fed lactose for 17 months displayed almost a **7-fold increase** in aortic sudanophilia (reflective of lipid deposition) compared to baboons fed equivalent quantities of sucrose[15]. Furthermore, gross atheromatous lesions were present in ~83% of baboons fed lactose, compared to 33% of baboons fed sucrose[15]. Therefore, it is remarkable that no study has assessed the causal effect of milk sugar in postprandial lipaemia and cardiovascular health in humans.

Galactose ingestion exaggerates postprandial triglyceride concentrations and produces a metabolic milieu indicating similarities to fructose metabolism. Our preliminary data are the first to demonstrate that the addition of galactose to a high-fat meal results in almost a **two-fold elevation** in postprandial triglyceridaemia compared to glucose ingestion (**Figure 1**). This demonstrates that ingesting milk sugars increases the exposure of the vascular system to atherogenic lipoproteins. Since the triglyceride response to galactose that we observed appears to be similar in magnitude to that of fructose[10], we explored whether galactose also reflected fructose

ingestion in other metabolic aspects. Indeed, similar to fructose ingestion[10], our preliminary data demonstrate that galactose ingestion increases plasma lactate concentrations and lowers plasma glucose and insulin concentrations compared to glucose ingestion (**Figure 2**). The increase in lactate concentrations with fructose compared to glucose ingestion is due to relatively rapid and unregulated flux of fructose through hepatic metabolic pathways[16]. This results in fructose being rapidly converted into lactate, glucose, hepatic glycogen, and (via *de novo* lipogenesis) triglycerides[16]. Therefore, the evidence that galactose also increases postprandial plasma lactate concentrations (**Figure 2**) and enhances hepatic glycogen synthesis[17], suggests both hepatic galactose metabolism is more similar to fructose than glucose metabolism, and that hepatic *de novo* lipogenesis may be a contributory factor for the increase in triglyceridaemia. Furthermore, to explore whether hepatocytes are potentially involved in the exaggerated triglyceride response that we observed (as is the case with fructose), we studied intracellular triglyceride accumulation in hepatocytes exposed to glucose alone or combined glucose and galactose at physiologically relevant concentrations for the human liver[18]. The partial replacement of glucose with galactose increased intracellular triglyceride accumulation by ~20% in hepatocytes *in vitro* (**Figure 3**). This shows that human liver cells accumulate more lipid when exposed to galactose, which would be expected to have detrimental effects on cardiovascular disease risk in humans, and also implies that the liver could be involved in galactose-induced hypertriglyceridaemia. We therefore hypothesise that galactose ingestion also stimulates hepatic *de novo* lipogenesis to a greater extent than glucose ingestion.

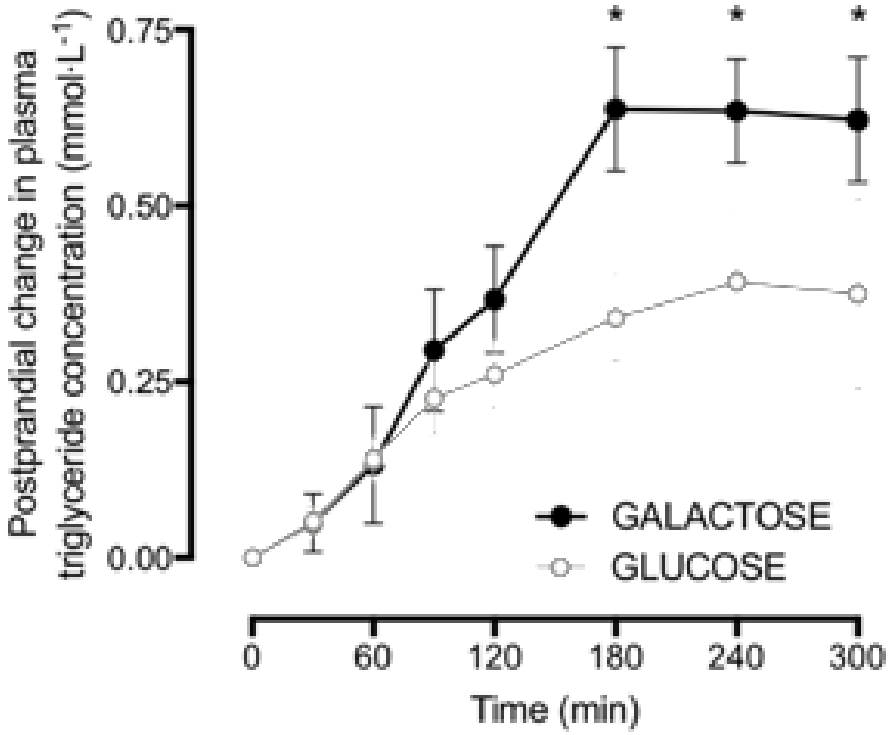

Figure 1. Postprandial triglyceride concentrations following ingestion of milk & cream (0.94 g fat·kg^{-1}) are exaggerated when co-ingested with additional galactose compared to glucose (both 0.75 g·kg^{-1}) in lean adults. *n* = 10. Data are means ± SEM. ***Unpublished data.***

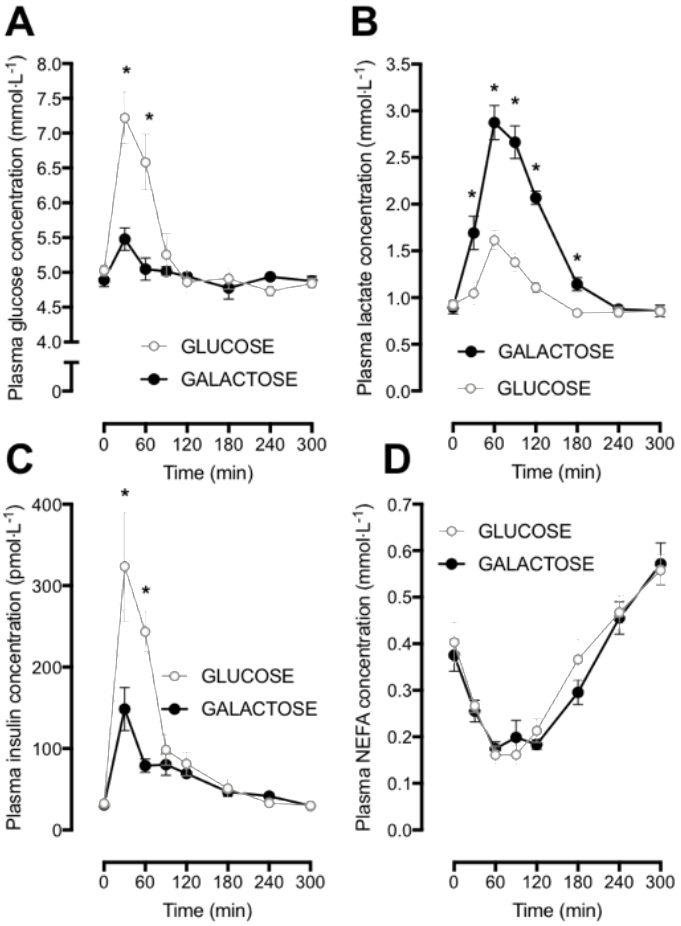

Figure 2. Postprandial glucose (**A**), lactate (**B**), insulin (**C**) and non-esterified fatty acid (**D**; NEFA) concentrations after ingestion of milk & cream (0.94 g fat·kg^{-1}), co-ingested with galactose versus glucose (both 0.75 g·kg^{-1}) in lean adults. *n* = 10. Data are means ± SEM. ***Unpublished data.***

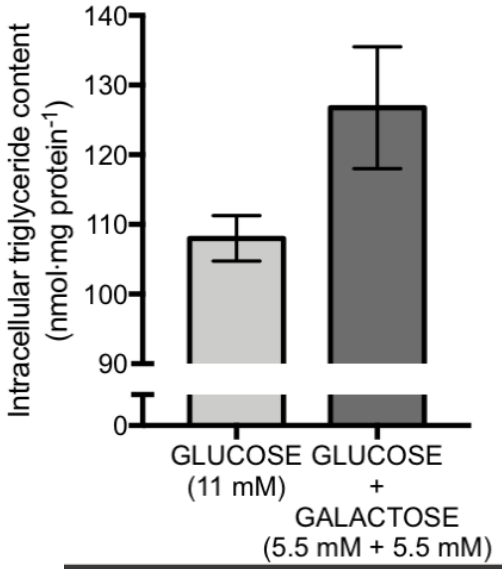

Figure 3. Intracellular triglyceride accumulation in hepatocytes is exaggerated when half of the glucose in incubation media is replaced with galactose. Importantly, these concentrations are physiologically relevant to hepatocytes in the postprandial state. Data are means ± SEM. ***Unpublished data.***

If lactose exaggerates lipaemia in similar ways to fructose-containing sugars, the public health consequences are substantial. Our preliminary data indicate that galactose ingestion exaggerates postprandial lipaemia via similar mechanisms to fructose ingestion[10]. If galactose and fructose exaggerate lipaemia via common mechanisms, an additive effect would have broad public health implications due to the common co-ingestion of sucrose (glucose-fructose) and milk products containing lactose. Furthermore, developing our understanding of milk sugars is timely, since milk drinks are currently not subject to the Soft Drinks Industry Levy, but former Chancellor of the Excequer, George Osbourne. has recently advocated the inclusion of milk-based drinks in this levy: "*I was already, before I left office, looking at whether you could extend it to sugar added to milk products like sugary milkshakes. I think it'll be for others to take further steps forward and I would predict those steps will be taken.*"[19]. Therefore, an additive effect of milk sugars and fructose-containing sugars on postprandial lipaemia would provide the necessary support for inclusion of milk-based drinks in the sugar levy, and would also inform whether intrinsic milk sugars should contribute to calculation of threshold(s) for the levy. The current thresholds are ≥5 g of free sugar per 100 mL and ≥8 g per 100 mL[20]. If lactose exaggerates lipaemia via common mechanisms to sucrose, then it would be logical to include milk sugars in the calculation of free sugars content for the determination of the levy threshold.

3. AIMS, OBJECTIVES AND HYPOTHESIS

PRIMARY AIM: To understand the effect and mechanism of lactose on postprandial lipaemia in men and women by addressing the following research objectives (RO):

RO1 - determine to what extent ingestion of lactose exaggerates postprandial triglycridaemia

RO2 - determine the effect of lactose ingestion on hepatic fatty acid synthesis (*de novo* lipogenesis)

RO3 - determine the degree to which lactose ingestion alters dietary fat oxidation

HYPOTHESIS: Lactose co-ingestion with a high-fat meal will exaggerate postprandial lipaemia and stimulate hepatic fatty acid synthesis (*de novo* lipogenesis) to a similar extent to sucrose ingestion, and a greater extent than glucose polymer co-ingestion, whilst suppressing dietary fat oxidation.

4. PROGRAMME AND METHODOLOGY

4.1. Study Design

This research will involve a randomised, double-blind, crossover study that will assess the acute postprandial metabolic responses to three different test drinks. All three test drinks will be comprised of a high-fat milkshake with the addition of either maltodextrin, sucrose or lactose. Individuals will be required to attend the laboratories for a preliminary visit, followed by the three laboratory trial days, with a washout period of 28±7 days.

4.2. Primary Outcome

The effect of lactose *versus* maltodextrin co-ingestion on postprandial incremental area under the curve (iAUC) for plasma triglyceride concentrations (6-hour postprandial period).

4.3. Secondary Outcomes

The effect of treatment (lactose versus maltodextrin co-ingestion on:

1) Postprandial *de novo* lipogenesis (*DNL*), using heavy water
2) Dietary fat oxidation, using a stable isotope-labelled palmitate

4.4. Tertiary Outcomes

Treatment (lactose *versus* fructose *versus* maltodextrin) by sex (male *versus* female) interaction effect on:

1) Postprandial incremental area under the curve (iAUC) for plasma triglyceride concentrations (6-hour postprandial period).
2) Postprandial de novo lipogenesis (DNL)
3) Dietary fat oxidation
4) Postprandial iAUC for plasma insulin concentrations
5) Postprandial iAUC for plasma glucose concentrations
6) Postprandial iAUC for plasma galactose concentrations
7) Postprandial iAUC for plasma fructose concentrations
8) Postprandial iAUC for plasma VLDL-rich triglyceride [Svedberg flotation rate (S_f): 20-400] concentrations
9) Postprandial iAUC for plasma chylomicron-rich triglyceride (S_f: >400) concentrations
10) Postprandial iAUC for plasma lactate concentrations
11) Postprandial iAUC for plasma non-esterified fatty acid concentrations
12) Postprandial iAUC for plasma beta-hydroxybutyrate concentrations
13) Postprandial iAUC for plasma uric acid concentrations

4.5. Participants and Eligibility Criteria

Twenty-four men (*n* = 12) and women (*n* = 12) will be recruited from the local area and matched for age and body mass index.

Inclusion Criteria:

Age: 18-50 years and premenopausal (for women);
Body mass index: 18.5 – 40 kg·m^2

Exclusion criteria:

weight instability (>5 kg change in body mass within last 6 months)
diagnosis of any form of diabetes
intolerances or allergies to any of the study procedures (e.g. lactose intolerance)
Galactose disorders (e.g. galactokinase deficiency, UDPgalactose-4-epimerase deficiency, galactose-1-phosphate uridyl transferase deficiency)
Fructose malabsorption
Inborn errors of fructose metabolism (e.g. fructokinase deficiency, aldolase B deficiency, fructose-1,6-bisphosphatase deficiency)
pregnant or lactating
any condition that could introduce bias to the study (e.g. diagnoses of lipid disorders, including cardiovascular disease, or therapies that alter lipid or glucose metabolism, such as statins or niacin).

4.6. Recruitment

Participants will be recruited through two different routes (1) direct advertisement and (2) letters sent to relevant patients in GP databases.

Direct Advertisement: Information about the study will be distributed via the placement of adverts and posters. Examples include: local newspapers and magazines; community noticeboards (e.g. in the public library, local market and cafes), community centres (e.g. leisure centres, churches, and art and music venues); larger local industry/establishments of employment (e.g. Wessex water and the University of Bath); healthcare centres (e.g. GP practices, pharmacies and dental clinics). In addition to printed adverts/posters, we will also utilise electronic routes: e.g. via E-mail bulletins, local community forums, university website/newsletters as well as social media (e.g. Twitter and Facebook). Potentially interested participants will be asked to contact the research team by email or phone if they want to find out more about the study. Potentially interested and eligible participants will be invited to a meeting to further discuss the study and what it entails. After reading the participant information sheets, if they decide to take part, they will be asked to sign an informed consent form.

Letters from GP database searches: Recruitment via this route will be conducted with the support of the Clinical Research Network (CRN) in BANES and Wiltshire. We will ask GP practices to search databases for patients who potentially meet the inclusion criteria. All patient identification and database searching will be conducted by the patient's existing care team (GPs, nurses and IT managers in study practices). Potentially eligible patients will be approached by a letter from their GP, jointly signed by the chief investigator. The recruitment letter will emphasise that participation is entirely voluntary and will not have any impact on their health care or their relationships with NHS staff.

Potentially interested participants will be asked to contact the research team via email or phone. Potentially interested and eligible participants will be invited to a meeting to further discuss the study and what it entails. After reading the participant information sheets, if they decide to take part, they

will be asked to sign an informed consent form. GP's will send out reminder letters to all eligible participants after two weeks, with an acknowledgement in the letter to ignore the reminder for those individuals who have already responded.

4.7. Screening

Participants will be invited to the University of Bath to provide informed consent, and thereafter will be invited to undertake the assessments of eligibility listed below. Given the range of screening measurement required, these measurements may take place over more than one visit. As all potential participants will be offered feedback from the screening, in the event that a potential participant be deemed ineligible for the study based on a single measurement, the individual will be informed immediately and the subsequent screening measures will only be carried out at the participant's request.

General health status: Suitability to participate (e.g. menopausal status, pregnancy etc.) and other practical screening questions (e.g. any reason why participants could not complete the study within a reasonable time-frame etc.) will be self-reported by the participant via a health screen questionnaire. Food allergies and intolerances will be reported in this questionnaire to ensure that participants are able and willing to consume the test drinks.

Anthropometrics: Body mass will be measured using digital scales and height using a stadiometer. Body mass index (BMI) will be calculated by dividing body mass (kg) by height (m) squared. Waist circumference (cm) will be measured at the narrowest point between the lowest rib and iliac crest, and hip circumference (cm) will be measured at the widest point of the gluteal using a tape measure.

Body composition (DXA): The participant will be positioned supine in a dual-energy x-ray absorptiometry (DXA) scanner with their extremities within limits indicated and not touching their torso if possible. Fat mass (kg), lean mass (kg), body fat percentage (%) and bone mineral density (g/cm^2) will be measured. Fat mass index will be calculated by dividing fat mass (kg) by height (m) squared.

4.8. Schedule of Measurements

Following screening participants will undertake the three main laboratory trial visits in a random order (randomized by JG using www.randomizer.org). Men and women will be allocated to separate randomization plans to allow for sex-difference comparisons to be made. The washout period between trials will be between 28±7 days.

Pre-trial standardisation
To assist in producing a similar metabolic state on the morning of laboratory trial days, participants will be asked to record diet and physical activity for 48 h before the first trial, and to replicate these for the 48 h before each subsequent trial. Physical activity levels will be confirmed by objective measures using an Actiheart physical activity monitor (Cambridge Neurotechnology Ltd). This is attached to the chest either using ECG pads or a chest strap for measurement of free-living physical activity[21].

Laboratory trial days
On the evening before trial days, a 5-mL blood sample will be taken (via venepuncture) for analysis of baseline ^2H enrichment of the body water pool. Participants will then consume a standardised meal with 3 $g \cdot kg$ body $water^{-1}$ of ^2H_2O for the assessment of fasting hepatic DNL[22]. Participants will continue to consume ^2H_2O throughout trial days for the measurement of postprandial DNL[22].

On postprandial study days, participants will arrive at the laboratories in a rested, overnight fasted state, after 24-hours of diet and physical activity standardisation.

Body mass will first be recorded, and then a cannula will be inserted retrograde into a pre-heated dorsal hand vein for arterialised blood sampling, and baseline blood and expired breath samples will be taken for analysis of plasma metabolite and hormone concentrations, plasma tracer enrichments, and indirect calorimetry, respectively. Participants will then consume a high-fat test drink containing 50 g of fat, plus 100 g of either **lactose, sucrose or maltodextrin (Table 1)**. The test drink will also contain 200 mg [U-^{13}C]palmitate in order to trace the fate of the dietary fat[22]. This quantity of lactose is roughly equivalent to 400 mL of milk and 400 g plain yoghurt, which is very high but not supraphysiological in light of typical UK intakes.

Table 1. Main laboratory trials and drink composition.

Trial	Carbohydrate type mixed with fat source (100 g)	Fat Source (50 g)
Maltodextrin	Glucose polymer; a common sugar substitute (control)	30 g palm oil and 20 g olive oil
Lactose	Galactose-glucose disaccharide (intervention)	30 g palm oil and 20 g olive oil
Sucrose	Fructose-glucose disaccharide (active comparator)	30 g palm oil and 20 g olive oil

Low-calorie-sweetened chocolate flavouring will be added to all drinks.

Participants will be advised that dairy products are used in this study and people following a vegan diet may therefore choose not to participate.

Blood samples will be drawn at 30, 60, 120, 180, 240, 300 and 360 min after ingestion of the test drink and will be centrifuged for plasma collection and determination of metabolite and insulin concentrations (**Figure 4**). A portion of plasma will undergo density gradient ultracentrifugation to obtain the VLDL-rich [Svedberg flotation rate (S_f): 20-400] and chylomicron-rich (S_f: >400) fractions[10]. Expired breath samples will be taken at 60, 120, 180, 240, 300 and 360 min after ingestion for indirect calorimetry (Douglas bag method) and [^{13}C] appearance in expired CO_2 (by isotope ratio mass spectrometry).

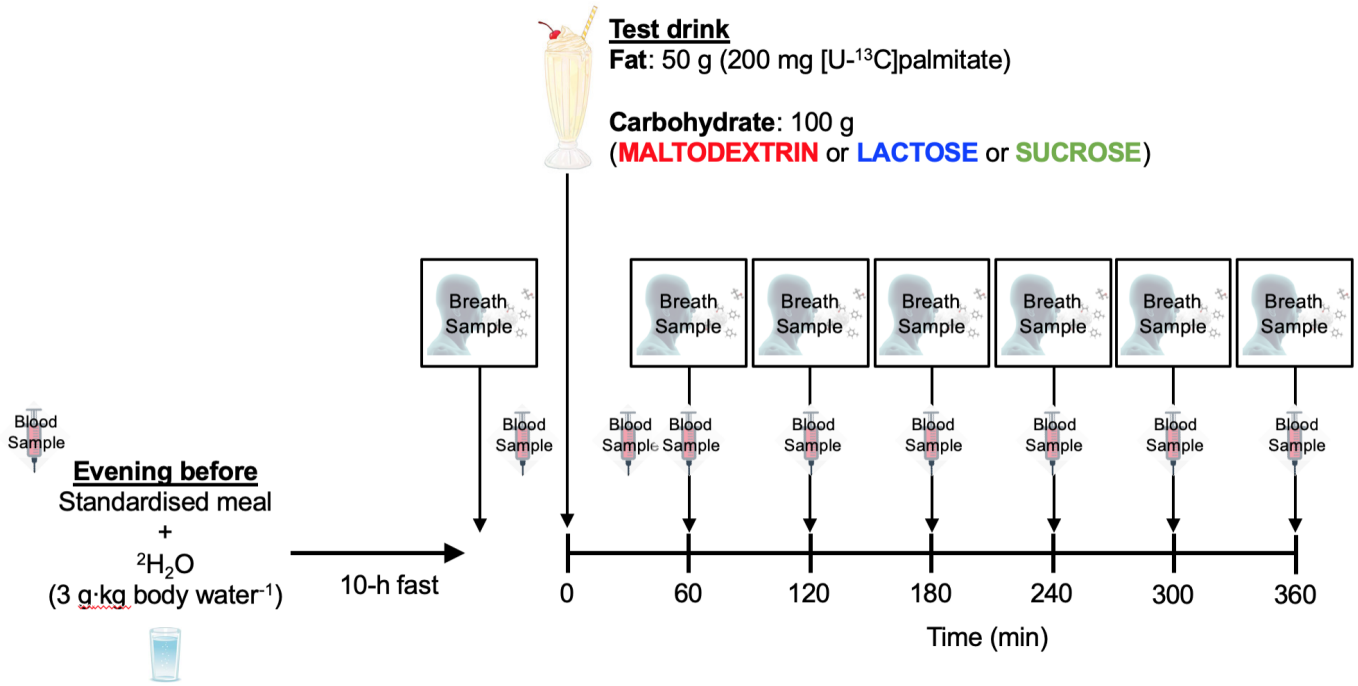

Figure 4. Schematic of the trial days

4.9. Measurements and Analysis

Plasma metabolite and hormone concentrations

Plasma concentrations of triglycerides, glucose, fructose, galactose, lactate, non-esterified fatty acids, glycerol, beta-hydroxybutyrate and uric acid concentrations will be determined by commercially-available enzymatic/colourimetric assays. Plasma insulin, estradiol and progesterone concentrations will be determined by commercially-available enzyme-linked, immunosorbent assays. A portion of plasma will undergo density gradient ultracentrifugation to obtain the VLDL-rich [Svedberg flotation rate (S_f): 20-400] and chylomicron-rich (S_f: >400) fractions[10].

Fasting and postprandial *de novo* lipogenesis

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Postprandial DNL will be determined by the incorporation of deuterium from ^2H_2O in plasma water into VLDL-TG palmitate using gas chromatography-mass spectrometry (Finnigan GasBench II Thermo Fisher Scientific, Paisley, U.K.) with monitoring ions with mass-to-charge ratios of 270 (M+0) and 271 (M+1) as we have previously performed[22]. In this respect, DNL refers to the proportion of newly synthesized palmitate in VLDL-TG, and this represents the synthesis of fatty acids from non-lipid precursors. The benefit of this method over tracing labelled lactose is that labelling the ingested sugar only captures the conversion of that specific precursor to fatty acids. The ^2H_2O method on the other hand, captures DNL from all precursors. Therefore, if lactose ingestion stimulates the conversion of lactate, galactose and glucose to lipid, this would be captured by our proposed method, but would be partially missed if using lactose labelling.

Substrate oxidation

Whole-body carbohydrate and fat utilisation rates will be determined in the fasted and postprandial state using indirect calorimetry (10-min samples every 60 minutes). Dietary fat oxidation will be determined by the appearance of [^{13}C] in breath CO_2 in line with our previous work[22]. Specifically, ^{13}C-to-^{12}C ratios in breath samples, and the relative rate of whole-body meal-derived fat oxidation will be calculated. First, the rate of expiration of $^{13}CO_2$ in breath will be calculated by multiplying CO_2 production rate as determined by indirect calorimetry (mmol·min^{-1}), by the expired CO_2

enrichment of ^{13}C (tracer-to-tracee ratio). To allow for sequestration of label into the bicarbonate pool, a dietary acetate recovery factor of 51% will be applied[23], and data will be corrected for fat-free mass as determined by DXA.

4.10. Sample Size

Based on our preliminary data, co-ingestion of galactose increased postprandial plasma triglyceride incremental area under the curve by a further 46.51 ± 0.28 mmol·L^{-1} x 300 min (mean ± SD) above glucose. Using this effect size (*d* = 0.75), 24 participants would provide a >90% probability (power = 0.94) of detecting such an effect with an α-level of 0.05 using a one-way, repeated-measures ANOVA and accounting for post-hoc comparisons of three factors by the principle of closed testing. Accordingly, 24 adults will be recruited. **Dropouts will be replaced in a rolling fashion to ensure a complete sample size of 24. Based on our previous experience of similar studies, we expect a dropout rate of ~20% and thus anticipate recruitment of ~28 people to achieve this target.**

4.11. Statistical Analyses

All of the analyses subsequently described will first be conducted on the entire sample as a primary analysis (males and females combined). A secondary analysis will include the analysis of subgroups (males only and females only), in addition to exploring sex-by-treatment interaction effects. The distribution of data will be assessed by visual inspection of Q-Q plots in addition to the Shapiro-Wilk test.

<u>Primary analyses</u>
Summary statistics (e.g. iAUC, AUC, peak concentrations) will be analysed by one-way ANOVA. Time-course data will be assessed by a two-way (time x treatment) ANOVA. Paired t-tests will be used for post-hoc comparisons, which will be corrected for multiple comparisons using the Holm-Bonferonni adjustment.

<u>Secondary analyses</u>
The analysis of each subgroup (males only and females only), will be analysed by one-way ANOVA, for summary statistics. Time-course data will be assessed by a two-way (time x treatment) repeated measures ANOVA. Paired t-tests will be used for post-hoc comparisons, which will be corrected for multiple comparisons using the Holm-Bonferonni adjustment.

To assess sex-by-treatment interaction effects, summary statistics will be analysed by a two-way (sex x treatment) mixed-model ANOVA. Time-course data will be analysed by three-way (sex x treatment x time) mixed-model ANOVA. Unpaired t-tests will be used for post-hoc comparisons, which will be corrected for multiple comparisons using the Holm-Bonferonni adjustment.

4.12. Storage and Management of Data

Blood samples will be partially processed on the day of collection before freezing in preparation for analysis. Throughout this period and during the subsequent analysis, blood and breath samples will be kept in a locked laboratory and will be inaccessible to anyone outside of the research team. Samples will be coded and identified only using anonymized sample identifier numbers. After analysis, samples will be stored until the work has been published. We will ask participants to consent to their samples being used in any other ethically approved project during the lifetime of the current project.

Some analyses will be required to be performed at other sites (e.g. University of Oxford for Mass Spectrometry). These samples will be coded and identified only using anonymized sample identifier numbers for shipment.

If a participant wishes to withdraw from the study, they will be permitted to do so, however their samples will only be destroyed by specific request of the participant. Withdrawing participants will be reminded of this at the point of withdrawal, and should they not wish that their samples not be analysed or further stored, said samples will be destroyed immediately.

Any results published from this study will be anonymous and include no identifiable information. An Excel data sheet maintained on a University computer will be used to record participant information and results. The computer will be password protected to ensure only the designated researchers can access the data.

Whilst all measurements included in the protocol provide insight into physiology and health, of those that have recognised clinical significance and established normal ranges, only bone mineral density from DEXA can be provided in a clinically meaningful timeframe. As such, should a participant's data for this measure fall outside clinically normal ranges, we will inform the participant's GP directly by way of a letter, provided the participant has consented for us to do so.

4.13. Expenses and Benefits

Reasonable travel expenses for visits to the University will be reimbursed on production of receipts or via claims for mileage. Participants will also be offered a voucher (e.g. Amazon/Love2shop gift vouchers) with a value of £150 upon completion of the study. Participants will be provided with an information pack containing their results for relevant outcomes along with reference ranges (e.g., physical activity level, diet, body composition, blood tests).

5. DISADVANTAGES/RISK AND DISCOMFORT

Participants will be asked to give up their time for repeated visits to undertake various tests. For some people this may be seen as an inconvenience. Blood samples will be collected using an intravenous catheter, which might cause minor discomfort during the procedure and may result in minor bruising. More serious complications are the very small risk of infection. However, the occurrence of such events is very rare and risks are further minimised by our strict adherence to best practice and standard operating procedures.

We will measure fat mass, fat-free mass, and bone mineral density, using dual-energy x-ray absorptiometry (DXA). DXA scans are non-invasive radiologic projection scans that use very low radiation, often compared to the exposure to background radiation experienced on daily basis in the UK. For the sake of the present study, this equates to less than one day of background radiation (and a tiny fraction of the amount of radiation experienced during a typical X-ray). These techniques are routinely used in hospitals and with elite athletes but nonetheless they do represent some exposure to a small amount of radiation.

We will utilise stable isotope tracers to determine key aspects of postprandial metabolism. These stable isotopes are non-radioactive and therefore pose no long-term risk to the health of the participants. On the evening prior to the study day, the participants will consume a loading dose of $^{2}H_2O$ (heavy water). The loading dose of $^{2}H_2O$ given is the amount to achieve a plasma water enrichment of 0.3%. This will be split into two equal doses with a ~2 h gap between consumption. This will decrease the likelihood of the participant feeling "dizzy" which can occur within ~1 hour

after consuming the water. Individuals prone to travel sickness appear to be more likely to get "dizziness" after consumption of water, but this dizzy feeling is transient and not harmful.

The above will be explained to the potential participant verbally and in the participant information sheet to ensure that they are fully informed before giving consent.

6. REFERENCES

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