

The effect of dietary carbohydrate manipulation on physical activity and other energy balance components: Study protocol for a 12-week randomised controlled trial

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Abstract

Carbohydrates are a staple component of modern diets. When classified by structure, the smallest chain carbohydrates are referred to as sugars. Free sugars comprise approximately 10-20% of energy intake in European populations. Global health guidelines advocate reducing free sugars intake below 5% of energy intake. It is unclear what impact these guidelines will have on physical activity and energy balance. Little is known about the effects of total (sugar and non-sugar) carbohydrate intakes on physical activity and energy balance too, therefore this study aims to measure the behavioural and metabolic responses to manipulating the type and amount of dietary carbohydrate in humans. Sixty humans, age 18-65 years, with a body mass index between 18.5-29.9 kg·m⁻² will be recruited for a randomised controlled trial. Participants will be randomised to a control diet (MODSUG), a low-sugar diet in line with public health guidelines (LOWSUG), or a low-carbohydrate ketogenic diet (LOWCHO) for 12-weeks. Self-reported dietary intake and objectively-measured physical activity will be monitored throughout the intervention. Participants will undergo laboratory testing at baseline, week 4, and week 12 of the diet. This will comprise muscle and adipose biopsies, a submaximal incremental treadmill protocol, measures of body composition, resting metabolic rate, and a mixed-meal tolerance test. Faecal samples will be obtained throughout the intervention to measure changes to the gut microbiome. This research will provide evidence to inform public health policy on the consumption of free sugars in healthy adults and will provide insight into the physiological effects of a low-carbohydrate ketogenic diet.

Introduction

Carbohydrates are a staple component of modern diets, which reportedly comprise nearly 50% of energy intake in the United Kingdom (roughly 224 grams per day) (Roberts et al., 2018). Dietary carbohydrates are commonly classified according to their size and structure as mono- or disaccharides (1-2 monomers), oligosaccharides (3-9 monomers), or polysaccharides (more than 9 monomers) (SACN, 2015). The smallest chain carbohydrates (e.g. glucose, fructose, sucrose) are commonly referred to as 'sugars', whereas polysaccharides are comprised of many molecules (e.g. amylose, amylopectin). Some of the sugars in foods can be defined as 'free sugars', which are 'all monosaccharides and disaccharides added to foods by the manufacturer, cook or consumer, plus sugars naturally present in honey, syrups and unsweetened fruit juices' (Swan, Powell, Knowles, Bush, & Levy, 2018). Free sugars by this definition reportedly comprise ~11% of energy intake in the United Kingdom (57 grams per day) (Roberts et al., 2018), and intake across developed nations has been reported to vary between 8% and 22% of energy intake (Wittekind & Walton, 2014). Both carbohydrates and free sugars are commonly ingested by many humans in developed countries, and therefore an understanding of the metabolic and energy balance effects of dietary carbohydrates and sugars is relevant to a wide population.

When referring to the intake of free sugars, this can often be considered as the co-ingestion of glucose and fructose (as sucrose or table sugar). Common sources of sucrose intake in Europe are soft drinks (sugar sweetened beverages), along with fruit juices, fruits, cakes and dairy products (Sluik, Engelen, & Feskens, 2015), therefore providing rationale for the specific targeting of sugar sweetened beverages with the Soft Drinks Industry Levy in the UK (Barber, 2017). Public health guidelines across the

world advocate that energy intake from free sugars should make up no more than 5% of an individual's total energy intake (Erickson, Sadeghirad, Lytvyn, Slavin, & Johnston, 2017; SACN, 2015; World Health Organisation, 2015). There are various reasons these guidelines may be beneficial for public health. There is reasonably strong evidence (despite a lack of randomised controlled trials) that dietary sugar intake is associated with the prevalence of dental caries (Freeman, 2014; Moynihan & Kelly, 2014). Data around the effects of free sugars on metabolic health and body weight are less conclusive. Increasing dietary sugar intake causes an increase in energy intake (SACN, 2015). However, increasing (or decreasing) dietary free sugars results in modest increases (or decreases) in body weight and isoenergetic exchange of free sugars with other sources of carbohydrates does not result in weight change (Morenga, Mallard, & Mann, 2013). Current evidence suggests that the increased weight observed with increasing free sugar intake is predominantly due to an energy surplus, via increasing energy intake. One rationale for reducing sugar intake, therefore, is that this would create an energy deficit which would lead to weight loss over time. However, an increase in energy intake without a clear change in body mass indicates that there must be interactions with other aspects of energy balance. Current evidence does not consider the effect of any compensatory changes in energy expenditure. On the balance of current evidence, advice to reduce total sugar intake seems sensible but a more complete understanding of the role of sugar intake on the complex and intricate components of energy balance is warranted.

In recent years, the notion that carbohydrates *per se* are detrimental to metabolic health has become prominent. Whilst the rate of absorption is dependent on the type and matrix of carbohydrate, from a physiological perspective, ingestion of glucose as a monomer or a polymer can be considered physiologically similar stimuli because hydrolysis of glucose polymers is not thought to be rate-limiting to intestinal absorption ((Gonzalez, Fuchs, Betts, & van Loon, 2017), suggesting the overall carbohydrate dose is an important consideration for general health outcomes. Furthermore, it is well-established that carbohydrate availability dictates the capacity to perform physical work (Krogh & Lindhard, 1920), but the role of carbohydrate in regulating physical activity behaviours has only recently been considered. Participants randomised to consume a carbohydrate-rich breakfast display an increase in 24-hour physical activity

energy expenditure (PAEE) compared with those randomised to remain fasted until midday (Betts et al., 2014). The magnitude of this difference is greatest prior to midday, near to when carbohydrate had been ingested and when glucose uptake to peripheral tissue is increased (Betts et al., 2014). This points towards a stimulatory role of carbohydrate or sugar on PAEE when carbohydrate is readily available to peripheral tissue. The amount of carbohydrate present in skeletal muscle is largely dictated by the amount of carbohydrate in the diet (Bergstrom, Hermansen, Hultman, & Saltin, 1967). As physical activity is performed by skeletal muscle, dietary carbohydrate intake may be an important regulator of physical activity behaviour.

Studies in which carbohydrate has been manipulated and physical activity has been measured have not been sufficient in answering whether manipulating dietary carbohydrate leads to a change in PAEE (Smith, Gonzalez, Thompson, & Betts, 2017). Often self-report measures of physical activity are used, which are not sufficiently sensitive to discern meaningful differences. Studies which have measured physical activity objectively, i.e. using pedometers (Foraker et al., 2014) or accelerometers (Layman et al., 2009; Tay et al., 2014), are confounded by a lack of information about actual carbohydrate intake or concurrent prescription of exercise interventions. More recent evidence using doubly-labelled water suggests that carbohydrate restriction increases total energy expenditure during weight maintenance after weight loss (Ebbeling et al., 2018), however this effect was influenced by the methods of analysis (Hall & Guo, 2018). The authors originally planned to use pre-weight loss energy expenditure as a baseline, but changed this to post-weight loss energy expenditure (Hall & Guo, 2018). The use of doubly-labelled water is inappropriate to measure total energy expenditure when either the body water pool changes by >3% or the average respiratory exchange ratio is unequal to the food quotient for the duration of the measurement (International Atomic Energy Agency, 2009). Ebbeling et al. (2018) used an assumed respiratory quotient based on the food quotient (i.e. macronutrient composition) of each participant (Black, Prentice, & Coward, 1986), but this did not incorporate changes which occur during weight loss. One study which investigated the effects of carbohydrate availability *per se* on energy expenditure using doubly labelled water in 5 participants suggested that physical activity was reduced on a low carbohydrate diet (7% carbohydrate, 83% fat, 10% protein) compared with a high

carbohydrate diet (83% carbohydrate, 7% fat, 10% protein) (Bandini, Schoeller, & Dietz, 1994), which points towards a potentially stimulatory role of carbohydrates to PAEE. To address these issues, studies using an objective method of measuring PAEE, which allow for incorporation of changes in substrate oxidation, are required.

Government targets to reduce sugar intake below 5% of total energy intake are not aimed at overall carbohydrate intake *per se*. In the breakfast study mentioned, sugar intake was significantly greater amongst individuals who ate breakfast compared with individuals who fasted until midday (Betts et al., 2014). As such, it is plausible that a regulatory role of carbohydrate on PAEE may be due to the type of carbohydrate rather than the absolute amount. Therefore, when assessing the potential role of carbohydrates on physical activity, it is important to consider the potential role of both carbohydrate type and amount. Therefore, we designed a study to investigate the role of carbohydrate **type** and **amount** on energy balance and physical activity, which addresses some of the limitations of previous research by objectively measuring free-living PAEE, incorporating changes in substrate oxidation which occur with altering carbohydrate availability. The purpose of this study is to measure the behavioural and metabolic responses to manipulating the type and/or amount of dietary carbohydrate in humans over a 12-week period. We approach this question from an integrated physiological perspective. We are interested in energy balance, metabolism, endocrinology, appetite, the gut microbiome, and food preference. The primary aim is to investigate the effects of dietary carbohydrate manipulation on PAEE, using combined accelerometry and heart rate monitoring, calibrated to each participant at each time of measurement, based on substrate oxidation and resting metabolic rate. This is the first study to objectively measure free-living energy expenditure incorporating changes in substrate oxidation in response to carbohydrate manipulation. The intervention arms are relevant from a public health perspective, with a direct investigation of the effects of implementing current public health guidelines on sugar intake. This research will provide evidence to inform public health policy on the consumption of free sugars in healthy adults, and will provide insight into the physiological effects of a low-carbohydrate ketogenic diet.

Methods

Study overview

Sixty metabolically healthy men and women, age between 18-65 years, will be recruited to participate in a randomised controlled trial with three arms. All data collection will be conducted in human physiology laboratories at the University of Bath. Following free-living assessment of habitual diet and physical activity level, participants will be randomised to adhere to one of three diets for a 12-week period in which the dietary carbohydrate content and/or type is manipulated. The macronutrient composition the intervention diets will aim for is as follows:

MODSUG (CONTROL) – 50% CHO (20% SUG), 15% PRO, 35% FAT

LOWSUG – 50% CHO (**<5% SUG**), 15% PRO, 35% FAT

LOWCHO – **<8% CHO*** (**<5% SUG**), 15% PRO, **≥77% FAT**

*or ≤50 g CHO per day

A schematic of progression through the study is presented in Figure 1. Participants will undergo three laboratory testing periods in total: at baseline, at week 4, and at week 12 of the diet. Diet and physical activity will be monitored for 7 days prior to randomisation, then at week 4 and week 12 of the intervention. Each laboratory test will comprise two laboratory visits on consecutive days for measures to be taken. On the first laboratory visit, participants will undergo muscle and adipose biopsies,

followed by a submaximal, incremental treadmill protocol to measure substrate oxidation and calibrate the activity monitor. The following morning, participants will return to the laboratory to undergo measures of body composition, resting metabolic rate, and a mixed-meal tolerance test. Participants will be provided with education on macronutrients and will be provided with guidance and feedback on which types of foods will help them achieve the macronutrient composition of the prescribed diets. Participants will also receive partial reimbursement of food expenses to help them achieve desired dietary changes. Whilst macronutrient composition will be manipulated, energy intake will not be prescribed.

[INSERT FIGURE 1 HERE]

Outcome measures

The primary outcome measure is physical activity energy expenditure (expressed as kJ per day).

Secondary outcome measures include time spent and energy expenditure at different intensities, weight, steps, energy intake, macronutrient intake, body composition and bone mineral density (using dual x-ray absorptiometry), waist:hip ratio, blood pressure, substrate oxidation, resting metabolic rate, interstitial glucose concentrations, fasting and postprandial metabolite and hormone concentrations, protein glycation, gene and protein expression in muscle and adipose tissue, muscle glycogen concentrations, urinary ketone concentrations, gut microbiome measures, urinary metabolomics, subjective appetite, and preference for food types. These are outlined in Figure 2 and will be assessed subject to funds available.

[INSERT FIGURE 2 HERE]

Participant characteristics and eligibility

The following are criteria for inclusion in the study:

- Body mass index 18.5-29.9 kg·m⁻²
- Age 18-65 years
- Able and willing to provide informed consent and safely comply with study procedures
- Females to maintain record of regular menstrual cycle phase/contraceptive use
- No anticipated changes in physical activity during the first 4 weeks of the study (e.g. holidays or training programmes)

The following are criteria for exclusion from the study:

- Any reported condition or behaviour deemed either to pose undue personal risk to the participant or introduce bias
- Any diagnosed metabolic disease (e.g. type 1 or type 2 diabetes)
- Any reported use of substances which may pose undue personal risk to the participants or introduce bias into the experiment
- Lifestyle not conforming to standard sleep-wake cycle (e.g. shift worker)
- Any reported recent (<6 months) change in body mass ($\pm 3\%$) (Stevens et al., 2006)
- Use of antibiotic medication in the last 3 months
- Use of prebiotic or probiotic products in the last month

Recruitment and enrolment

Participants will be recruited from the University of Bath campus and the local community by word of mouth, by poster advertisement, and by social media. Participation will be centred on the provision of obtaining informed consent prior the study and throughout participation. Participants will be sent an information sheet after expressing interest in the study and will be asked to read thoroughly. Where possible, researchers will talk through this information sheet in person. Participants will be asked to take time to consider and ask questions about the study over the following week. Pre-enrolment, participants will undergo a consultation to affirm the information

provided in the information sheet and provide clarity on the procedures that the participant will undergo as part of the study. Once this information has been provided and consent has been clarified verbally, participants will be asked to initial and sign an informed consent form. Following this, participants will undergo eligibility screening by completing a health questionnaire. We will assess body mass index to make sure they meet this criterion. They will be reminded that their consent is completely voluntary and will be reminded of their right to withdraw from the study procedures at any point without providing justification.

Preliminary testing

Once participants are formally enrolled on the study, they will visit the laboratory to begin their preliminary measures. Participants will be asked to fast for ~4-5 hours prior to visiting the laboratory to minimise interference with respiratory measures (Compher, Frankenfield, Keim, Roth-Yousey, & Evidence Analysis Working, 2006). Height will be measured using a stadiometer (Seca Ltd., Birmingham, UK) in the Frankfurt plane with shoes removed. Body mass and bioelectrical impedance will be measured using digital scales (Tanita, Amsterdam, The Netherlands) with participants barefoot and wearing light clothing. Hip and waist circumference will be measured using a handheld tape measure (Seca Ltd., Birmingham, UK).

Resting metabolic rate will be measured using the Douglas bag technique. Participants will rest in bed in a recumbent supine position for ~10 minutes. When rested, the participant will be given a mouthpiece and nose clip to wear, and three 5-minute samples of expired air will be collected into Douglas bags. Researchers will enter the room to change the Douglas bag at the end of each sample and note down ambient conditions. The expired fraction of oxygen and carbon dioxide will be entered into equations by Frayn and Jeukendrup & Wallis (Frayn, 1983; Jeukendrup & Wallis, 2005) to calculate resting metabolic rate (RMR) and substrate oxidation. The fractions of inspired oxygen and carbon dioxide will be measured concurrently to correct for the dynamic ambient conditions (Betts & Thompson, 2012). Detailed methods for indirect

calorimetry are outlined below in the '**Indirect calorimetry**' section. Then participants will be asked to complete a food preference task on a laptop. The details of which are outlined in the '**Food preference task**' section.

Participants will then complete a 20-minute treadmill walk to calibrate the physical activity monitor to their substrate oxidation and energy expenditure, based on a protocol adapted from Brage et al. (Brage et al., 2007). The walk will comprise four 5-minute stages at 5.2 km·h⁻¹ with progressive inclines of 0%, 3%, 6% to 9% with participants wearing a heart rate monitor (Polar, Warwick, UK). During the last minute of each stage, heart rate will be recorded and expired breath will be collected into a Douglas bag to measure energy expenditure and substrate oxidation.

Following the treadmill walk, participants will receive an Actiheart monitor (CamNtech Ltd., Cambridge, UK). This is a small chest-worn device which records heart rate, inter-beat-interval, and physical activity energy expenditure. Participants will be shown how to wear apply the device. They will be asked to keep it on for a 7-day period. This will provide a free-living measure of energy expenditure. Participants will be provided with a food diary and a set of portable weighing scales. They will be asked to record all food and drink they ingest for a 7-day period coinciding with the 7-days of wearing the Actiheart monitor. This will be used to evaluate macronutrient composition and will provide an estimate of energy intake. The 7 days of actiheart and food diary will be repeated in the 7 days prior the 4-week and 12-week laboratory tests during the intervention. Participants will also be provided with a pedometer (3DTrisport, Realalt, UK) which will be worn throughout the week of habitual monitoring and the 12-week intervention.

Once participants have completed their week of habitual lifestyle monitoring and laboratory testing has been arranged, they will be provided with a faecal collection kit and asked to collect a faecal sample within 24 hours of visiting the lab. The kit will contain 1 pair of disposable gloves, 1 "faeces catcher" paper, 1 collection pot, 1 plastic Ziploc bag, 1 icepack and 1 small opaque cool bag for discreet transportation, along with a detailed instruction sheet advising them how to collect the sample. Participants will be advised to place the collection pot inside the Ziploc bag, and place this inside

the opaque cool bag to store in their fridge at 4 degrees, and then transport the sample to the lab in the cool bag together with frozen icepacks. During the week-long monitoring periods before each laboratory visit.

Participants will be fitted with a research-grade continual glucose monitor (CGM) (Freestyle Libre, Abbott, UK) which will collect data for ~14 days. A member of the research team will fit the continual glucose monitor and this will remain attached to the participant until being removed by a member of the research team. A new CGM will be fitted at 10 weeks into the intervention to record data between week 10 and 12. In total, interstitial glucose will be measured for 7 days of habitual lifestyle, the first 7 days of the diet intervention, and the final 14 days of the diet intervention.

Randomisation

Participants will be randomised to one of three diet interventions by a member of the research team who is not participant-facing. Allocation will be stratified on two levels: by sex (male vs female), and mean physical activity level (PAL) across the habitual monitoring period (<1.70 vs ≥ 1.70). PAL is simple way of assessing an individual's habitual physical activity, and is calculated by taking total energy expenditure and dividing by basal metabolic rate. A PAL ≥ 1.70 is associated with lower risk of many metabolic diseases including type 2 diabetes and cardiovascular disease (FAO/WHO/USU, 2001). Due to the nature of the intervention, dropouts are expected. As such, we will be conducting a rolling recruitment to achieve statistical power whereby dropouts will be replaced by the next recruited individual. A dropout will be defined as an individual who does not wish to continue with the diet intervention within the first 4 weeks (i.e. initial 4 weeks will be a completer's analysis). When the first 4 weeks have been completed, any deviations in adherence to diets will be considered as part of the research question (i.e. intention to treat analysis).

Laboratory tests

There are three laboratory tests across the study, one at baseline, one at week 4, and one at week 12. These require laboratory visits which will be split across two days. Day one will take place in the afternoon ~16:30 and day two will take place the

following morning ~08:00. On day one, participants will be asked to arrive at the laboratory following a minimum 5-hour fast and they will be asked not to perform structured exercise in this period. They will undergo an abdominal adipose tissue biopsy. The abdominal region will be sterilised with iodine and local anaesthetic (lidocaine hydrochloride) will be administered. After 5 minutes, when the area is numb, a 14 G needle attached to a 50 mL syringe will be inserted into the subcutaneous abdominal adipose tissue. A vacuum will be created using the syringe and adipocytes aspirated into the syringe via the needle. Pressure will be applied to stop any bleeding then the wound will be dressed. Treatment and analysis of samples is outlined in the section titled '**Adipose biopsy samples**'.

Participants will then be given time to rest before any other procedures are carried out. When the participant is ready and willing, they will undergo a muscle biopsy from the vastus lateralis. The participant's quadriceps will be sterilised with iodine before local anaesthetic (lidocaine hydrochloride) is administered subcutaneously and on the muscle fascia. When the area is numb, a small incision will be made to the skin and muscle fascia using a sterile scalpel blade. Then a Bergstrom needle will be inserted into the muscle belly and ~2-3 snips will be made with suction applied, as described by Tarnopolsky et al. (Tarnopolsky, Pearce, Smith, & Lach, 2011). The cutaneous incision will then be stitched up and pressure will be applied to the site. Muscle sampling handling and analyses is outlined in the section titled '**Muscle biopsy samples**'.

Following the muscle biopsy, participants will be allowed time to recover and rest. They will then complete a graded exercise protocol on the treadmill. This will mirror the Actiheart calibration protocol outlined in the '**Preliminary measures**' section, with four 5-minute stages where the speed of the treadmill will be fixed but the gradient will be increased. Participants will be asked to walk at 5.2 km·h⁻¹ at 0%, 3%, 6%, and 9% incline. During the last minute of each stage heart rate will be measured using a heart rate monitor (Polar, Warwick, UK), substrate oxidation and energy expenditure will be measured by collecting expired air in Douglas bags, and ratings of perceived exertion (RPE) will be measured using Borg's 6-20 scale (Borg, 1970). Participants will be

asked to have a relaxing evening at home ready for the continuation of the laboratory testing the following morning.

On day two, participants will be asked to arrive to the laboratory at around 08:00, following an overnight fast of 10-12 hours. They will be asked to drink a pint of water between waking and attending the laboratory and will be asked to refrain from performing physical activity during their commute (i.e. take the bus or car, instead of walking or cycling). Height will be measured using a stadiometer (Seca Ltd., Birmingham, UK), with participants barefoot in the Frankfurt plane. Body mass will be measured using digital scales (Tanita, Amsterdam, The Netherlands) with participants barefoot and wearing light clothing. Hip and waist circumference will be measured using a handheld tape measure (Seca Ltd., Birmingham, UK). Following this, participants will undergo a whole-body dual x-ray absorptiometry (DXA) scan to assess body composition and bone mineral density.

At ~08:45, participants will be escorted to the resting laboratory where they will rest in a recumbent supine position for 15 minutes. When rested, the participant will be given a mouthpiece and nose clip to wear, and three 5-minute samples of expired air will be collected into Douglas bags. Researchers will enter the room to change the Douglas bag at the end of each sample and note down ambient conditions. The expired fraction of oxygen and carbon dioxide will be entered into equations by Frayn and Jeukendrup & Wallis (Frayn, 1983; Jeukendrup & Wallis, 2005) to calculate resting metabolic rate (RMR) and substrate oxidation. The fractions of inspired oxygen and carbon dioxide will be measured concurrently to correct for the dynamic ambient conditions (Betts & Thompson, 2012). Detailed methods for indirect calorimetry are outlined in the ***'Indirect calorimetry'*** section.

Following this, a measure of blood pressure will be taken using an automated sphygmomanometer (Diagnostec EW3106, Panasonic, Japan). A cannula will then be inserted into a dorsal hand or antecubital vein and the hand of the corresponding arm will be placed in a box heated to 55°C to arterialise the blood (Edinburgh et al., 2017).

Two mL of blood will be drawn to make sure blood is flowing from the cannula and then it will be flushed with sterile saline solution (B. Braun, Pennsylvania, USA) to maintain patency. Whilst waiting for the vein to arterialise, the participant will complete visual analogue scales (VAS) for appetite and mood; these are 100-mm scales which are attached to a statement with opposing extremes (e.g. not at = 0, extremely = 100). Following this, participants will complete a food preference task; details of which are outlined in the section titled '**Food preference task**'. Once this is complete, a baseline blood sample will be collected. For all blood samples, 2 mL blood will be drawn and disposed of, 10 mL blood will be collected for analysis, and then the cannula will be flushed with 5-10 mL of sterile saline solution (B. Braun, Pennsylvania, USA) to maintain patency. All samples will be dispensed into 2 sterile collection tubes. One containing ethylenediaminetetraacetic acid (EDTA) (Sarstedt, Nümbrecht, Germany) which will be immediately centrifuged to extract blood plasma. The other containing plastic beads (Sarstedt, Nümbrecht, Germany) which will be allowed to clot at room temperature for 15 minutes before being centrifuged to extract blood serum. All samples will be centrifuged at 4000 g for 10 minutes at 4°C. Blood serum and blood plasma will be aliquoted into Eppendorf tubes (Eppendorf, Hamburg, Germany) and stored on dry ice for the remainder of the visit, before being moved and stored at -80°C until analysis.

Following the baseline blood sample, participants will be provided with a mixed meal tolerance test (MMTT). Participants will ingest a milk chocolate milk shake (Ensure Plus, Abbott, Illinois, US) equating to 30% of resting metabolic rate as determined by indirect calorimetry in the first laboratory test (e.g. 600 kcal for an individual with a resting metabolic rate of 2000 kcal·day⁻¹). The composition of this drink is similar in composition to the control diet and is reflective of typical food intake of European populations; the contribution of macronutrients to total calories are 54% CHO (23% SUG), 31% FAT, 15% PRO. A timer will be started when the participant ingests the first sip of the drink and they will be asked to try and finish ingesting the MMTT within 5 minutes. Blood will be collected at 15, 30, 45, 60, 90, 120, 150, 180, 210, and 240 minutes following ingestion of the MMTT. VAS measures will be collected each hour minutes following ingestion of the MMTT. A 5-minute expired breath samples of will be collected at each hour following ingestion of the MMTT to measure dietary induced

thermogenesis. Blood pressure will also be measured each hour. Participants will be asked to remain in a semi-recumbent supine position for the duration of the 240 minutes, unless they need to use the toilet, and will be allowed to perform sedentary tasks like watching television, reading, or working on a laptop. Participants will be allowed to take their hand out of the heated box until 10 minutes prior each blood sample to ensure the vein remains adequately arterialised. Towards the end of the postprandial period, participants will be asked to complete a second food preference task. Then, the cannula will be removed and pressure will be applied to prevent bruising. Urine produced during the laboratory visit will be collected into a beaker for measurement of urea nitrogen excretion, relevant for indirect calorimetry measures.

Indirect calorimetry

Energy expenditure and substrate utilisation will be determined using equations from Frayn and Jeukendrup & Wallis (Frayn, 1983; Jeukendrup & Wallis, 2005), with adjustments for the contribution of glycogen during low-intensity exercise:

$$\text{Fat oxidation at rest and during exercise (g}\cdot\text{min}^{-1}\text{)} = (1.695 \times \text{VO}_2) - (1.701 \times \text{VCO}_2) - (1.77 \times \text{urinary nitrogen excretion})$$

$$\text{Carbohydrate oxidation at rest (g}\cdot\text{min}^{-1}\text{)} = (4.55 \times \text{VCO}_2) - (3.21 \times \text{VO}_2) - (2.87 \times \text{urinary nitrogen excretion})$$

$$\text{Carbohydrate oxidation during exercise (g}\cdot\text{min}^{-1}\text{)} = (4.344 \times \text{VCO}_2) - (3.061 \times \text{VO}_2) - (0.40 \times \text{urinary nitrogen excretion})$$

At rest, these equations assume that glucose provides all the carbohydrate for metabolism, whereas during low-intensity exercise carbohydrate metabolism is achieved by an equal contribution from glucose and glycogen. These equations will also be used with the assumption that the energy contents of fat, glucose, glycogen, and nitrogen are 9.75, 3.74, 4.15, and 4.09 respectively (Jeukendrup & Wallis, 2005).

488

489 ***Food preference task***

490 Participants will complete food preference tasks. These will comprise an 'alternative
491 forced choice task' and an 'ideal portion size task' (Wilkinson et al., 2012). The AFCT
492 consists of 18 plates of food which are individually photographed on a white plate or
493 transparent bowl. The participant will choose which food they would 'choose to eat
494 right now'. Foods are distinguished into three categories: sweet high-carbohydrate
495 foods, non-sweet high-carbohydrate foods, non-sweet low-carbohydrate foods. The
496 outcomes of this test will indicate preference for different types of foods corresponding
497 to the categories above. The ideal portion size task consists of 3 test foods: chocolate
498 mousse, roasted potatoes, and vegetarian sausages (corresponding to the 3
499 categories above). These are photographed in portions ranging from 20-1000 kcal in
500 increments. Participants can increase or decrease the amount of food they desire at
501 by being instructed to imagine they are offered this food right now, using the left and
502 right arrow keys to adjust the portion to show how much of it they would eat.

503

504 ***Adipose biopsy samples***

505 Samples will be cleaned using sterile saline solution (B. Braun, Pennsylvania, USA).
506 Cleaned adipose tissue will be aliquoted, weighed, and snap frozen in liquid nitrogen
507 before being stored at -80°C until further analyses. With the exact nature of the
508 analyses being dependent on the size of the sample, these samples will be assessed
509 primarily for gene expression (via real-time polymerase chain reaction) and protein
510 expression (via Western blotting). The sample required to perform these analyses are
511 ~50 and ~30 mg respectively.

512

513 ***Muscle biopsy samples***

514 Muscle tissue samples will be promptly snap-frozen in liquid nitrogen and stored on
515 dry ice until being transferred to -80°C awaiting analysis. Tissue will be prioritised for
516 glycogen analysis, which will be performed similarly to previous studies (Jansson,
517 1981; van Loon, Saris, Kruijschoop, & Wagenmakers, 2000). Muscle tissue will be

freeze-dried before removal of non-muscle fibre material. Dried muscle tissue will be heated in hydrochloric acid to hydrolyse glycogen to glycosyl units, before being neutralised, and glucose concentration will be analysed. Leftover tissue will be used to analyse gene expression (via real-time polymerase chain reaction) and protein expression (via Western blotting), with the precise nature of the analyses to depend on tissue sample size.

Faecal tissue handling and processing

Faecal samples provided by participants will be homogenised, aliquoted and stored frozen at -80°C for later processing. Total DNA will be extracted using a commercial kit (QIAGEN QIAamp® Fast DNA Stool Mini Kit) and faecal water will be extracted for metabolic profiling. Extracted DNA will be analysed to determine taxonomic composition and functional potential via 16S rRNA sequencing and shotgun metagenomic sequencing, respectively. A range of bioinformatic and statistical tools will be used to determine taxonomic and functional diversity of microbes present. No analyses will be conducted on the human components of the sequenced DNA. Faecal water will be prepared through centrifugation and both faecal water and faecal matter will be later processed using 'omic' technologies (e.g. mass spectrometry and nuclear magnetic resonance) to determine presence of metabolites. Analyses of faecal and urine samples collected will be conducted in Teagasc laboratories (Moorepark, Cork, Ireland). On some occasions the analyses may be done in collaboration with a third party including commercial companies. All samples and extracted DNA will be stored at -80°C for future analysis.

Implementing the intervention and monitoring adherence

Adherence to the study diets is a key component of the study and is one of the major shortcomings of previous research into carbohydrate manipulation and energy balance (Smith et al., 2017). There will be a focus on developing and maintaining a friendly but professional relationship between the research team and participants – easing them into the study procedures and familiarising them with the laboratories. To further facilitate adherence, participants will be reimbursed up to a predetermined

amount of £18 per week for participants in the MODSUG or LOWSUG groups, and £26 per week for participants in the LOWCHO group. This is due to the relative difficulty in adhering to low carbohydrate diets and the relative expense of typically suitable menus compared with the other groups. Members of the research team will meet with participants weekly and will keep in touch with them via email to check how they are finding the study. Participants will be asked to send a weekly measure of body weight using scales they have been provided (Etekcity Digital Scales, California, USA) and urinary ketone body concentrations in the fasted state (using Ketostix, Ascensia, Newbury, UK). Participants will be asked to record 3-day food diaries during each week. These will be analysed and feedback returned to the participant as soon as possible. Feedback about what they have done well in the previous week and where they might be able to improve to meet their macronutrient targets will also be provided. We will focus on specific food items and tailor any substitutions to habitual dietary habits.

The absolute cut-off of 50 g of CHO in the LOWCHO diet is according to recommendations from ketogenic diet proponents (Volek & Phinney, 2012). This will comprise 8% of energy intake if an individual's daily energy intake is 2337.5 kcal, with the assumption that 1 g of carbohydrate provides 3.74 kcal of energy. It is anticipated that, in most individuals, 50 g of carbohydrate will equate to $\leq 8\%$ of energy intake. However, in individuals consuming fewer kilocalories per day, the recommended grams per day of carbohydrate will be decreased to equate to 8% of energy intake (e.g. an individual consuming 1600 kcal per day will be asked to consume ~34 g of carbohydrate per day).

We will attempt to check the accuracy of information from food diaries by rearranging the energy balance equation, using measures obtained from DXA throughout the study, as validated previously (Racette et al., 2012; Sanghvi, Redman, Martin, Ravussin, & Hall, 2015). It is worth noting that the energy expenditure values will be extrapolated from the 7 days of recording to reflect the time between measurements. The energy content of the tissues may also be influenced by the change in water content induced by the intervention, particularly in the ketogenic LOWCHO diet.

Hydration status has been shown to influence measures of non-fat mass by DXA with little influence on fat mass measures (Toomey, McCormack, & Jakeman, 2017). For this reason, we will interpret rearranged energy balance data in the context of these limitations.

Diet analysis

There is no consensus on the exact caloric value of each macronutrient because fibre ingestion, water ingestion, energy balance, dietary macronutrient composition, dietary food variety, chewing during meals, meal timing/distribution, preparation/cooking of food, alcohol intake, physical activity level, sex, age, disease status, and stress are all thought to influence the metabolisable energy of foods (Sanchez-Pena et al., 2017). Further complicating individual variability in the energy harvested from macronutrients is the gut microbiome, particularly through the production of short-chain fatty acids (Canfora, Jocken, & Blaak, 2015). Despite these limitations, metabolisable caloric values for each macronutrient have been estimated in the literature, suggesting that the caloric values of carbohydrate, fat, protein, and alcohol are around 3.74, 9.75, 4.09, and 7.10 kcal·g⁻¹ (Jeukendrup & Wallis, 2005; Morgan & Levine, 1988). Whilst these exact values may be slightly contended amongst academics who may prefer to use values from older studies (Atwater, 1910; Frayn, 1983), they have been chosen because the same values have been used for oxidation of macronutrients during measures of energy expenditure. Diet analysis for preliminary measures, the 3-day food diaries for week 1-3 and week 5-11, and the 7-day food diaries from week 4 and week 12 will be analysed using diet analysis software (Nutritics, Dublin, Ireland). Grams for each macronutrient will be exported and these will be multiplied by the caloric factor mentioned. This will be used to provide energy in kilocalories and as a percentage of total energy intake. Total energy intake will not be fed back to participants, just macronutrient proportions as a percentage of total energy intake. One major limitation with current sugar guidelines is that they target 'free sugars', but there is no legal requirement for manufacturers to include this information, the requirement is to provide information on total sugars. Therefore, as we are unable to definitively determine free sugars in commercial products, we will use all sugars other than from fruits and vegetables as a proxy for 'free sugars'. We will report the following in our

results: carbohydrate, fat, protein, alcohol, total sugars, sugars from fruit and vegetable sources, all other sugars. This means our intervention will target total sugars minus fruit and vegetable sugars, as it is impossible to measure the intake of free sugars *per se*.

Laboratory test design

It is worth clarifying the rationale behind the test design. Obtaining muscle and adipose biopsies in the fasted and rested state is preferable, but obtaining biopsies immediately prior to the mixed meal tolerance test would risk these procedures influencing metabolic measures. The treadmill walk should also be performed in the fasted state, but there should be sufficient washout between the treadmill walk and the mixed meal tolerance test. Furthermore, it is inappropriate to perform the treadmill walk immediately before biopsies due to the potential effects this would have on gene and protein expression. Therefore, it was decided to perform the treadmill walk following the biopsy procedures, allowing for a rest period between the procedures and the walk. This enables calibration of the Actiheart monitor to each participant's individual physiology at each visit, which allows for fluctuations in substrate oxidation and resting metabolic rate across the course of the intervention. The treadmill walk acts to standardise physical activity levels the evening before the meal test, which reduces the chance of seeing metabolic effects which are caused solely by the previous evening's activity. Participants will attend the lab the morning after the biopsy and treadmill walk in an overnight fasted state having eaten dinner a of their choice at home. This allows assessment of body composition and the mixed meal tolerance test to be performed prior to lunchtime of the second laboratory testing day.

Statistical analyses and power calculation

A required sample size for the present study was estimated based on the Bath Breakfast Project (Betts et al., 2014) using G*Power 3.1 software (Faul, Erdfelder, Lang, & Buchner, 2007). The mean \pm standard deviation PAEE for the fasting vs breakfast groups during the morning (when differences in carbohydrate availability between groups were present) were 311 ± 124 kcal vs 492 ± 227 kcal. Based on this

effect size, a between-subject design with 20 participants in each group would provide an >85% chance (power) of detecting the expected effect with an α -level of 0.05. Therefore, 60 participants will be recruited. As dropout with diet interventions is expected to occur, a rolling recruitment model will be employed whereby dropouts are replaced to achieve a complete dataset. The sample size estimations have been based on the primary outcome of PAEE which, as the primary behavioural dependent variable, also displays the most variance and thus the metabolic variables may require fewer participants to achieve a similar power, given greater precision of measurement.

Descriptive statistics will be calculated on Microsoft Excel (Microsoft, Washington, USA). Two-way ANOVAs will be used to identify significant interactions between conditions and time across the study. Post hoc adjustments will be employed to determine the nature of these differences using GraphPad Prism (GraphPad Software Inc., California, USA). Both p-value hypothesis testing and inference-based statistics will be utilised. Where p-value hypothesis testing is used, significance will be accepted at $p = 0.05$.

UPDATED STATISTICAL ANALYSIS PLAN

For parallel group RCTs it is more efficient to use ANCOVA with baseline values as the covariate to estimate the causal effects of an intervention (i.e., estimated differences between interventions vs. control, see <https://doi.org/10.3945/ajcn.115.119768>). Therefore, the statistical analysis plan was adjusted accordingly in line with the following description. SPSS v25 (IBM, USA) were used for statistical analyses. Total (tAUC) and incremental area under the curve (iAUC) were calculated using the Time Series Response Analyser. Figures were drawn using Prism v9.5.0 (GraphPad Software Inc., USA). For all outcomes with quantitative units at week 4 and week 12, ANCOVA was used to assess differences between groups with baseline values as the covariate. Unadjusted means are presented for baseline outcomes, but ANCOVA-adjusted means (and mean differences vs MODSUG) are reported for week 4 and week 12 unless otherwise stated. Since skeletal muscle protein and adipose mRNA levels were expressed as the fold-change from baseline, one-way ANOVAs were used at week 4

and week 12 respectively to detect differences between groups. *Post-hoc* comparisons were made according to the principle of closed testing to assess the effect of sugar restriction (LOWSUG vs MODSUG) or ketogenic carbohydrate restriction (LOWCHO vs MODSUG). Data from Visual Analogue Scales were assessed by repeated-measures ANOVA of within-group comparisons due to their subjective nature. Figure legends state whether means or ANCOVA-adjusted means are presented for each outcome. Simple linear regression and Pearson correlation coefficients were used to assess linear associations between outcomes where appropriate. Significance was accepted at $p \leq 0.05$. Data are presented as mean and 95% confidence intervals (CI) unless otherwise stated.

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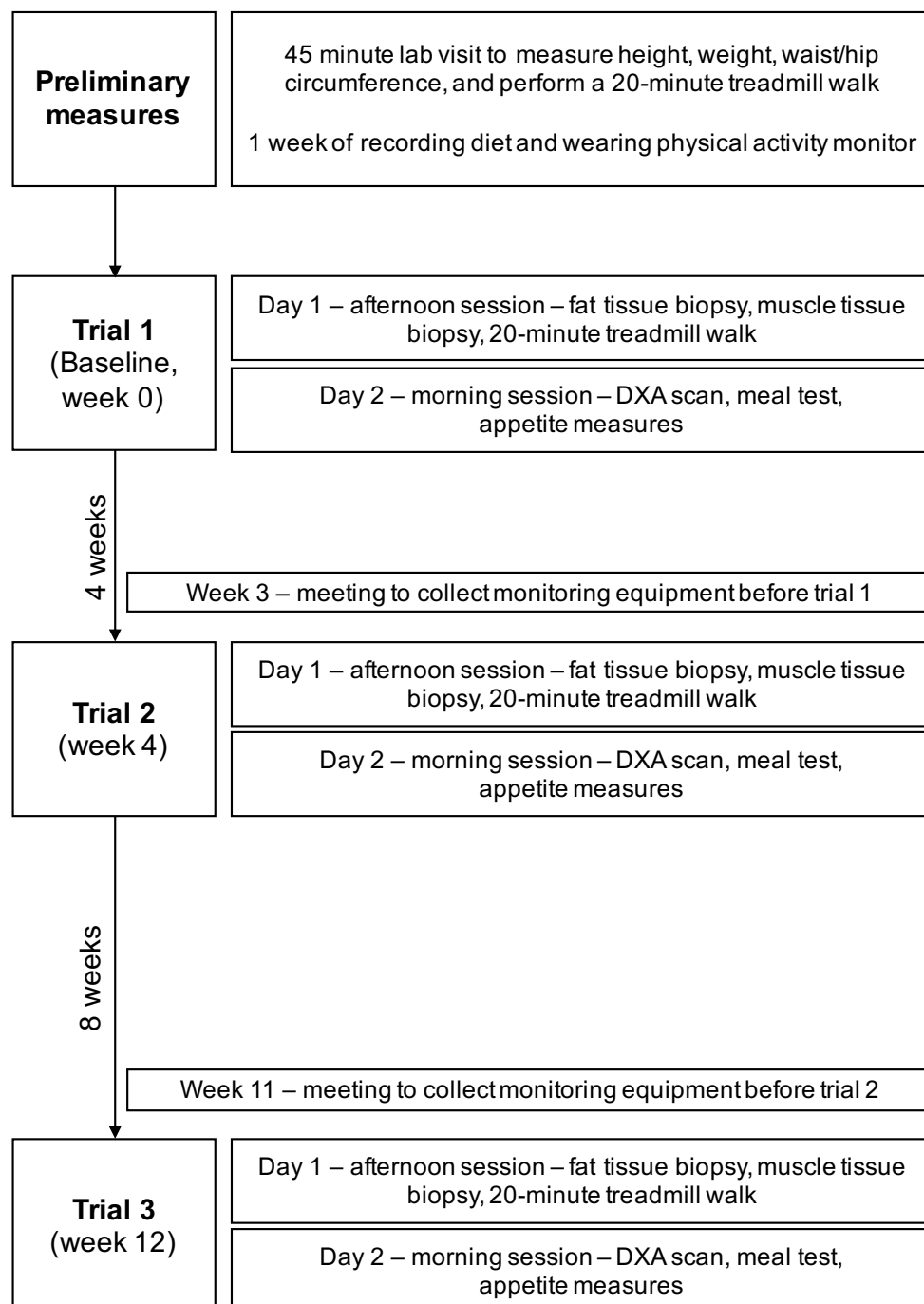
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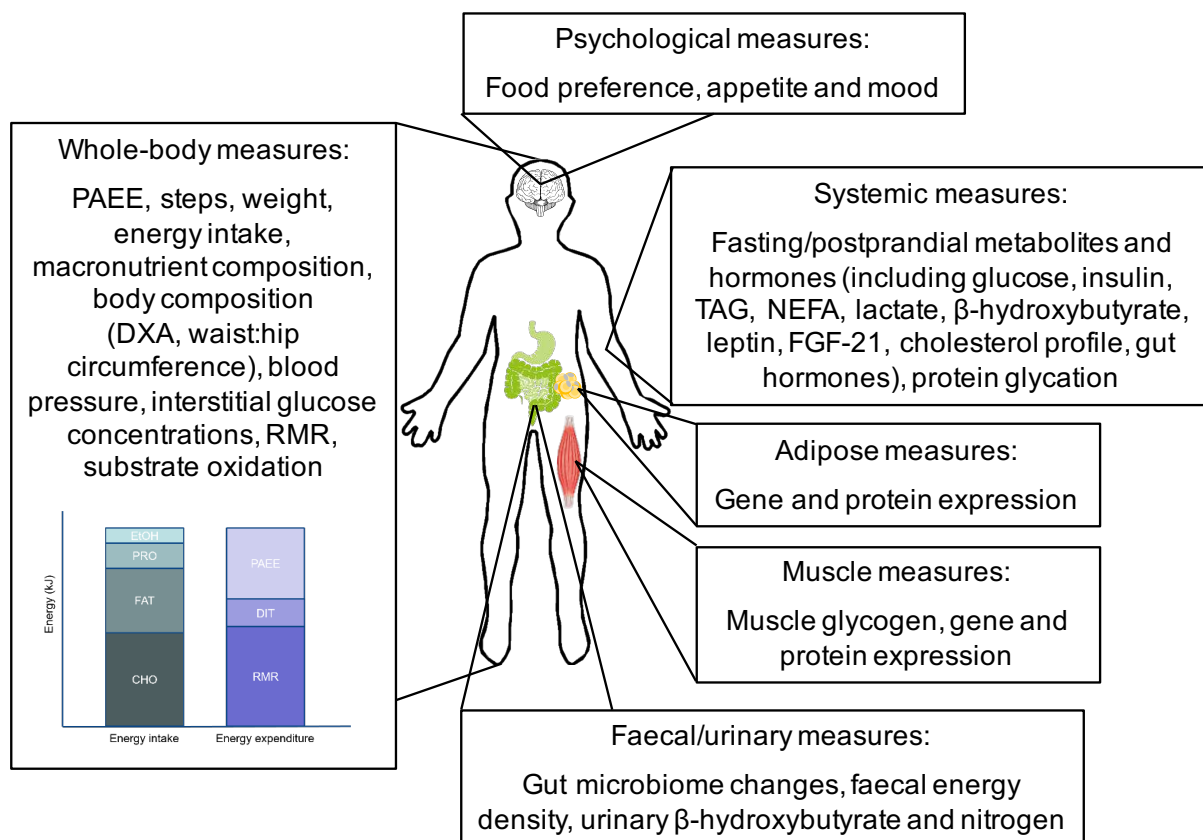
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Figures

Figure 1. Schematic of trial progression. Participants will complete preliminary measures and then be randomised to one of three trial arms. Laboratory visits will comprise three trials at baseline, week 4, and week 12 of the intervention. They will be split across two days, day one in the afternoon and day two the following morning. Physical activity and diet will be measured for 7 days during week 4 and during week 12. DXA = dual x-ray absorptiometry.



882 **Figure 2. Schematic of outcome measures in the study. PAEE = physical activity**
 883 **energy expenditure, DXA = dual x-ray absorptiometry, RMR = resting metabolic**
 884 **rate, TAG = triacylglyceride, NEFA = non-esterified fatty acids, FGF-21 =**
 885 **fibroblast growth factor 21.**



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