Protocol

Study title: Exploring true interindividual heterogeneity of CVD risk factors in response to low-carbohydrate diets using a replicate crossover randomised controlled trial.

Short title: Low-carbohydrate diet and LDL-c interindividual variability

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Sponsor: University of Bath

SIGNATURE PAGE

The undersigned confirm that the following protocol has been agreed and accepted and that the Chief Investigator agrees to conduct the study in compliance with the approved protocol and will adhere to the principles outlined in the Declaration of Helsinki, the Sponsor's SOPs, and other regulatory requirement.

I agree to ensure that the confidential information contained in this document will not be used for any other purpose other than the evaluation or conduct of the investigation without the prior written consent of the Sponsor

I also confirm that I will make the findings of the study publicly available through publication or other dissemination tools without any unnecessary delay and that an honest accurate and transparent account of the study will be given; and that any discrepancies from the study as planned in this protocol will be explained.

For and on behalf of the Study Sponsor:

Signature: Date:

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Name (please print):

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Position:

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Chief Investigator:

Signature: Date:

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Name: (please print):

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1. SYNOPSIS

Study Title	Revealing true interindividual heterogeneity of CVD risk factors in response to low-carbohydrate diets using a replicate crossover randomised controlled trial.
Short Title	Low-carbohydrate diet and LDL-c interindividual variability
Trial Design	Replicate Randomised Controlled, Crossover Trial in men and women.
Trial Participants	Men and women older than 18 years old, with BMI 18.5-30 kg/m^2, LDL-c <4.9 mmol/L, non-fasting triglyceride <2.3 mmol/L, and free from atherosclerotic cardiovascular disease or diabetes mellitus.
Intervention	Thrice 24 hours of each of the following diets: Typical UK diet: 50% carbohydrate, 35% fat (14% saturated), 15% protein (Control) Low-carbohydrate diet: 8% carbohydrate, 77% fat (31% saturated), 15% protein (Intervention) Food will be provided by research team
Planned Sample Size	20 (men and women)
Primary Objective	To quantify between-person variation in LDL-C responses to a low-carbohydrate diet, against the random within-person variation in LDL-C responses- revealing a path to precision use of low-carbohydrate diets.
Secondary Objectives	To quantify between-person variation in other cardiometabolic health responses to a low-carbohydrate diet, against the random within-person variation in responses of these outcomes. To characterise the atherogenicity of the responses to a low-carbohydrate diet between individuals, over and above LDL-C concentrations alone (lipoprotein particle size, number and composition, markers of insulin resistance and inflammation). To explore whether LDL-C responses are predicted by genotype, baseline biomarkers, age, physiological characteristics, and metabolic rate.
Primary Outcomes	Fasted LDL-c to establish whether there is true inter-individual heterogeneity in response to 24 hours of a low-carbohydrate diet.
Secondary Outcomes	Fasted glucose to establish whether there is true inter-individual heterogeneity in response to 24 hours of a low-carbohydrate diet.
Tertiary Outcomes	Other fasted metabolic health markers (low-carbohydrate vs control) helping elucidate primary outcome and potentially predictors of heterogeneity: • NMR fatty acid profile (i.e., mono and polyunsaturated, and saturated) • NMR Amino Acids (i.e., branched-chain amino acids and tyrosine) • NMR Ketone Bodies (i.e., beta-hydroxybutyrate, acetoacetate, acetone) • Plasma beta-hydroxybutyrate concentration • Urine acetoacetate • Plasma Lactate concentration • Plasma triglycerides concentration • Plasma glycerol concentration • Plasma NEFAs concentration • Plasma insulin concentration • Plasma C-reactive protein Potential atherogenicity of responses to a low-carbohydrate diet (low-carbohydrate vs control):

	• Plasma lipoprotein subclass profiling with lipid concentrations within 14 subclasses • Plasma sphingomyelin- and ceramide-rich particles Baseline potential predictor markers of LDL-c responses • Age • Weight • Body Mass Index (BMI) • Waist circumference • Hip circumference • Waist-to-hip ratio (WHR) • Body composition (e.g., fat mass, fat free mass) • Resting Metabolic Rate (RMR) • Concentrations of biomarkers of cholesterol synthesis • Concentrations of biomarkers of cholesterol absorption • 12 common LDL-c raising single nucleotide polymorphisms in 11 genes • LDL receptor mRNA content in PBMCs • LDL receptor protein content in PBMCs
Sponsor	The University of Bath
Principal Investigator	Bruno Spellanzon
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2. BACKGROUND AND RATIONALE

2.1. Executive Summary

Ketogenic diets are very low-carbohydrate diets that increase hepatic production of ketone bodies, which have several effects as substrates and signals[1,2]. Ketogenic diets are used to manage some conditions (e.g., drug-resistant epilepsy), and their potential for managing many other conditions (e.g., cancer) is being actively explored. Indeed, the British Dietetic Association acknowledge the potential for ketogenic diets to improve glycaemic control to a greater extent than some other diets in people with type 2 diabetes. A lower fasting glucose is also relevant to future mortality in people without diabetes[3]. Ketogenic diets are popular in the general population, with up to 15% of the UK following a low-carbohydrate diet (https://www.statista.com/forecasts/997894/diets-and-nutrition-in-the-uk). There is, therefore, great interest in ketogenic diets amongst people with and without health conditions.

While the potential for ketogenic diets to lower glycaemia is promising[4], there are possible risks for other aspects of cardiometabolic health. Specifically, these diets often result in large increases in concentrations of atherogenic lipoproteins. Data from a meta-analysis of randomised controlled trials (RCTs) indicated that a ketogenic diet can increase low-density lipoprotein cholesterol (LDL-C) concentrations by 1.08 mmol/L (95%CI: 0.37-1.79 mmol/L)[5]. Cumulative lifetime exposure to high LDL-C concentrations increases atherosclerotic cardiovascular disease risk. Conversely, a lifetime with 0.35 mmol/L lower LDL-C concentration (based on genetic variants in LDL-C) results in a lower

risk (odds ratio: 0.79, 95%CI: 0.76-0.81) of experiencing cardiovascular events[6]. Therefore, whilst there are some potential health benefits to a ketogenic diet, there are also some potential health risks. This heterogeneity in response to diets could justify personalisation and ensure the use of ketogenic diets for only individuals who can be confidently predicted to respond more favourably.

2.2 Relevant Literature and Pilot Data

Low-carbohydrate diets are popular but may have risks for some people

The most common motivation for individuals to follow a low-carbohydrate diet is weight loss, and with other potential benefits including avoiding hyperglycaemia[4,7]. However, these benefits could come with insidious risks, as such as increased atherogenic lipoprotein concentrations, although this risk seems to differ between people. Specifically, low-carbohydrate diets often increase low-density lipoprotein cholesterol (LDL-C) concentrations, with meta-analyses quantifying this at a clinically relevant mean increase of 1.08 mmol/L (95%CI: 0.37-1.79 mmol/L)[5]. Our pilot data demonstrate that low-carbohydrate diets can increase LDL-C concentrations at the same time as body mass is decreasing and glycaemia is falling (Fig-1).

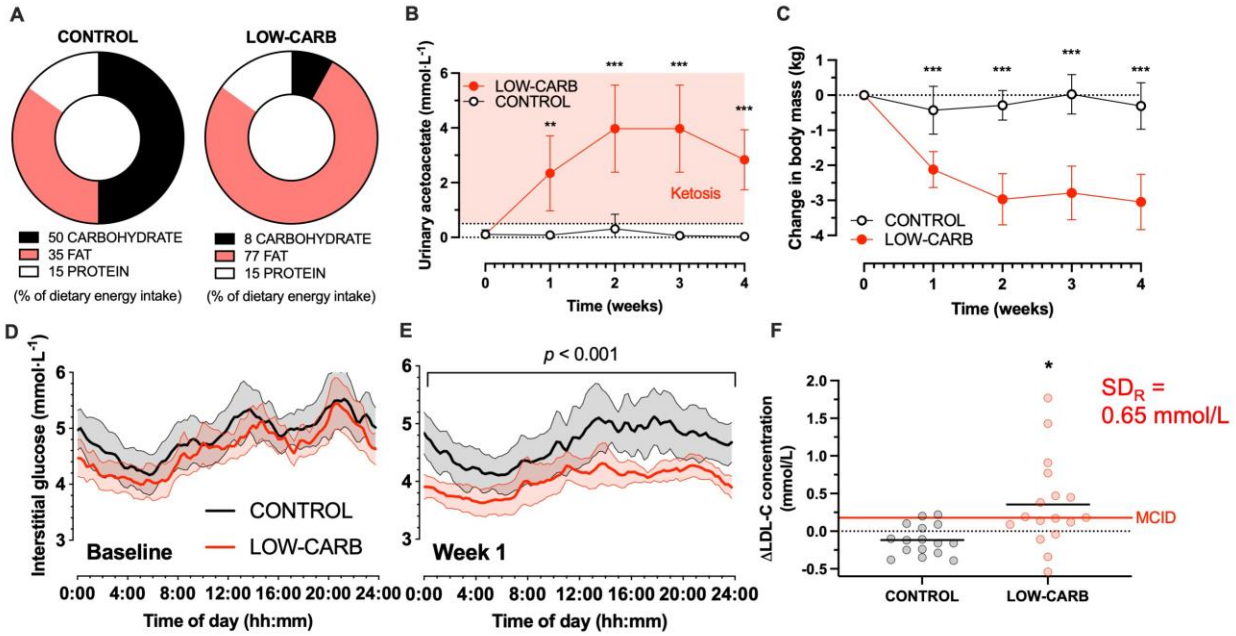

Figure-1. Thirty-four healthy adults were randomized to either control or low-carbohydrate (B; LOWCARB) diets for four weeks (A). Compliance to low-carbohydrate was confirmed with urinary acetoacetate concentrations (B). The low-carbohydrate diet decreased body mass (C) and diurnal glycaemia assessed with continuous glucose monitors (D and E). However, the low-carbohydrate diet also increased low-density lipoprotein cholesterol (LDL-C) concentrations on average, albeit with substantial heterogeneity (F). MCID, minimal clinical important difference; *, p<0.05 for LOW-CARB vs. CONTROL; ***, p<0.001 for LOW-CARB vs. CONTROL

Cumulative lifetime exposure to high circulating LDL-C proportionally increases atherosclerotic CVD risk[6]. Conversely, a lifetime with 0.35mmol/L lower LDL-C results in a lower risk of cardiovascular events (odds ratio: 0.79, 95%CI: 0.76-0.81)[6]. Therefore, whilst a substantial proportion of people are using low-carbohydrate diets for weight-loss and glucose lowering, these people could be increasing their future atherosclerotic CVD risk (moderated by body mass). There is, therefore, a need to establish if heterogeneous increases in atherogenic lipoproteins are meaningful, consistent and predictable, as a basis for personalising the safe use of low-carbohydrate diets (Fig-2).

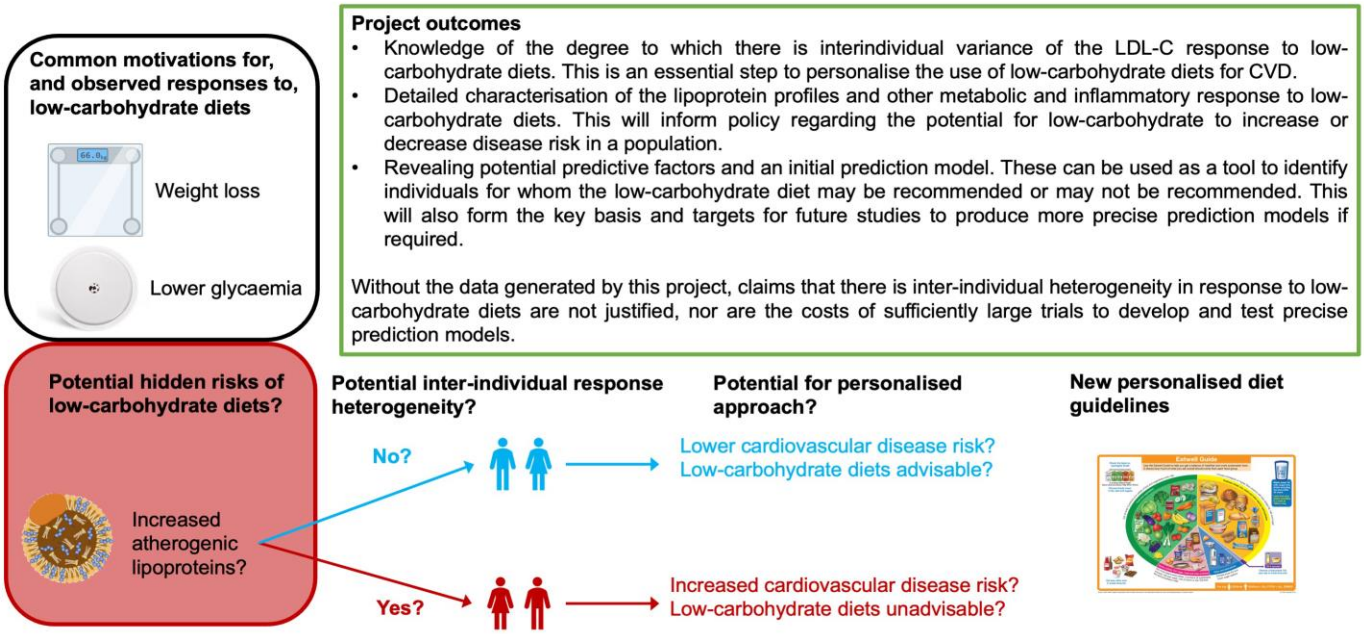

Figure-2. Weight loss and lower glycaemia are common motivations for, and observed responses to, low-carbohydrate diets in the general population. There could, however, be hidden risks of low-carbohydrate diets, particularly in some people. Some people may increase atherogenic lipoproteins which would increase cardiovascular disease risk, whereas others may obtain the benefits of low-carbohydrate diets without the risks. Project outcomes displayed top-right.

It may be possible to personalise the use of ketogenic diets.

Our preliminary data also suggest there is likely to be inter-individual heterogeneity in the LDL-C response to low-carbohydrate diets (Fig-1F). Importantly, the variance of change was higher with low-carbohydrate versus control, evidenced by the variance comparison standard deviation (SD_{IR}) being 0.65 mmol/L. This suggests possible clinically meaningful response heterogeneity to low-carbohydrate diets. Some people may show large increases in LDL-C when following a low-carbohydrate diet, whereas others may not show an increase in LDL-C at all. This notion is also supported by the RISCCI trials (https://clinicaltrials.gov/study/NCT03270527) making important advancements on this topic. However, parallel-group and single-period crossover RCTs are inadequate research designs to isolate the component of variation corresponding to participant- by-diet interaction relevant to investigate the possibility of true inter-individual variability in response to a particular diet[32]. We demonstrate this concept with a repeated period (replicate)-crossover of the 2-h glucose/insulin response to breakfast (Fig-3;[8]).

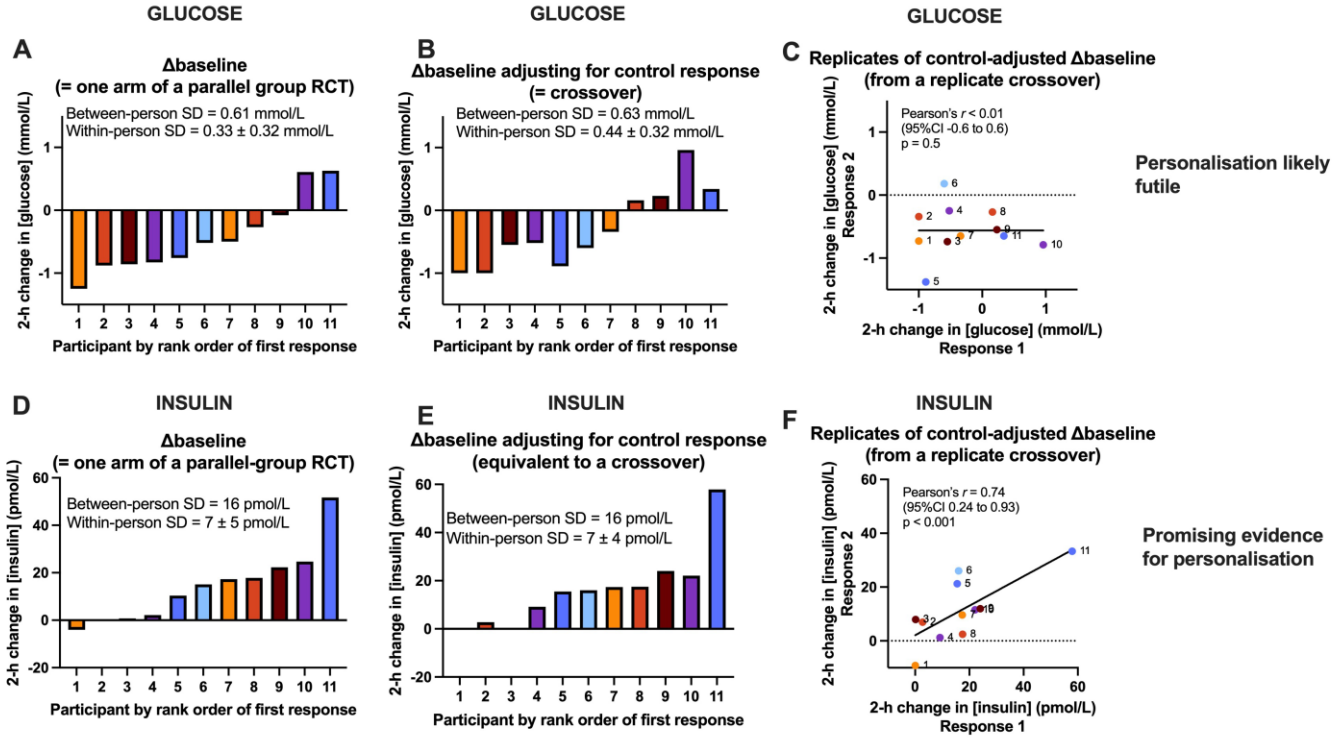

Figure-3. The 2-h change in glucose (A) and insulin (D) concentrations after a standardised breakfast (444 kcal porridge) in 11 healthy people with evidence that the between-person SD is larger than the within-person SD. Panels B and E represent the same data when taking into account the control response (extended overnight fasting for 2 h). However, only the replicate crossover can establish whether a response to a treatment (in this example, breakfast) displays reliable inter-individual heterogeneity. For glucose (C) the replicate crossover does not provide evidence of reliable inter-individual variability of response to breakfast, whereas for insulin (F) there is evidence of true inter-individual variability in response to breakfast consumption and therefore justification for personalisation. Data from https://doi.org/10.21203/rs.3.rs-4317405/v2

To establish effectiveness/harms for individuals requires randomisation and replication at the individual level (a replicate-crossover[9]). Only a replicate-crossover (an alternative form of n-of-1 trial) can estimate the component of variation corresponding to the participant-by-diet interaction required to establish inter-individual heterogeneity of response to diets (Fig-4). Without this, participant-by-diet interaction cannot be separated from within-person variance (Fig-4[9]).

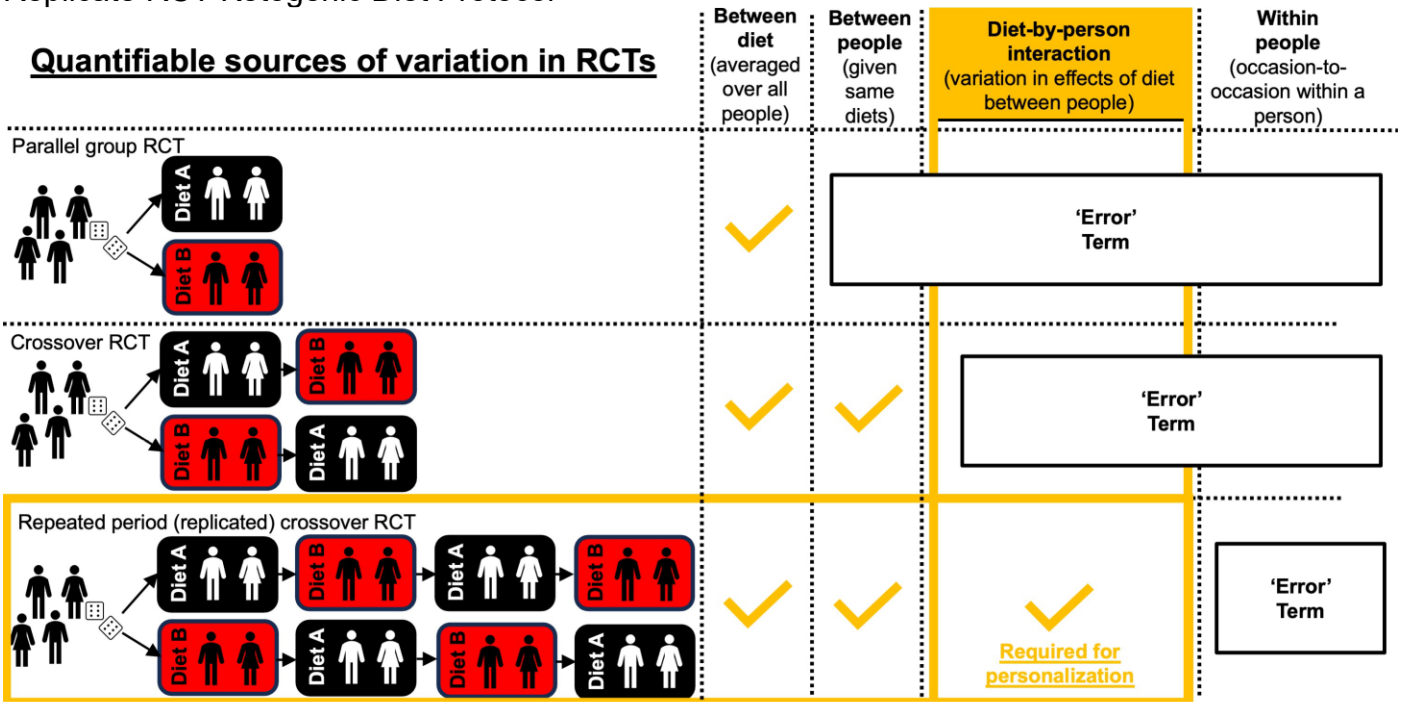

Figure-4. To provide personalised approaches to nutrition and disease prevention/management separation of the diet-by-person interaction from within-person variance is required, which can be done using a repeated period (replicated) crossover RCT. Based on Senn (2015) *Statistics in Medicine*. 'Error' term = residual variable in model.

Intervention Duration

A key requirement for the deployment of n-of-1 trials is that the outcome measures respond rapidly to the intervention and also washout rapidly with removal of the intervention[10]. Fortunately, LDL-C responses to (and washout from) low-carbohydrate diets are rapid. For example, in a second preliminary RCT we conducted, 25 individuals consumed control and low-carbohydrate diets in a crossover controlled-feeding design (Fig-5A). Remarkably, just 24 hours on a low-carbohydrate diet was sufficient to observe similarly sized responses to those observed in our 4-week parallel group RCT[11].

We observed marked ketosis (Fig-5B) and a mean increase in LDL-C concentrations (Fig-5C) of similar magnitude to the 4-week intervention. Importantly, we still observed a clinically meaningful SD_{IR} of 0.28 mmol/L for LDL-C even though macronutrient intakes and saturated-to-unsaturated fat ratios were identical across all participants. 50% of participants displayed a control-adjusted LDL-C increase in response to low-carbohydrate diets that was greater than the minimal important clinical difference (MCID), and 50% of participants displayed a control-adjusted LDL-C response to low-carbohydrate diets that was smaller than the MCID. This provides evidence that some individuals may show unfavourable responses for LDL-C whereas others may not show unfavourable responses, which are not explained by variation in diet composition (Fig-5C). Furthermore, the 24-h responses to a low-carbohydrate diet are consistent with longer-term responses that we and others have observed (Fig-5D). To address the aim of this project, the LDL-C response does not need to have fully plateaued but does need to represent the full long-term response. Based on these data, we have chosen a 24-h duration for each period to balance the ability to detect meaningful increases in LDL-C, maximising participant compliance and minimising dropout. Fortunately, the washout of LDL-C after low-carbohydrate diets is also as rapid as the increase during controlled-feeding of low-carbohydrate diets (Fig-5E[12]). This allows for an opportunity to study the heterogeneity of LDL-C responses to a ketogenic diet using a replicate crossover.

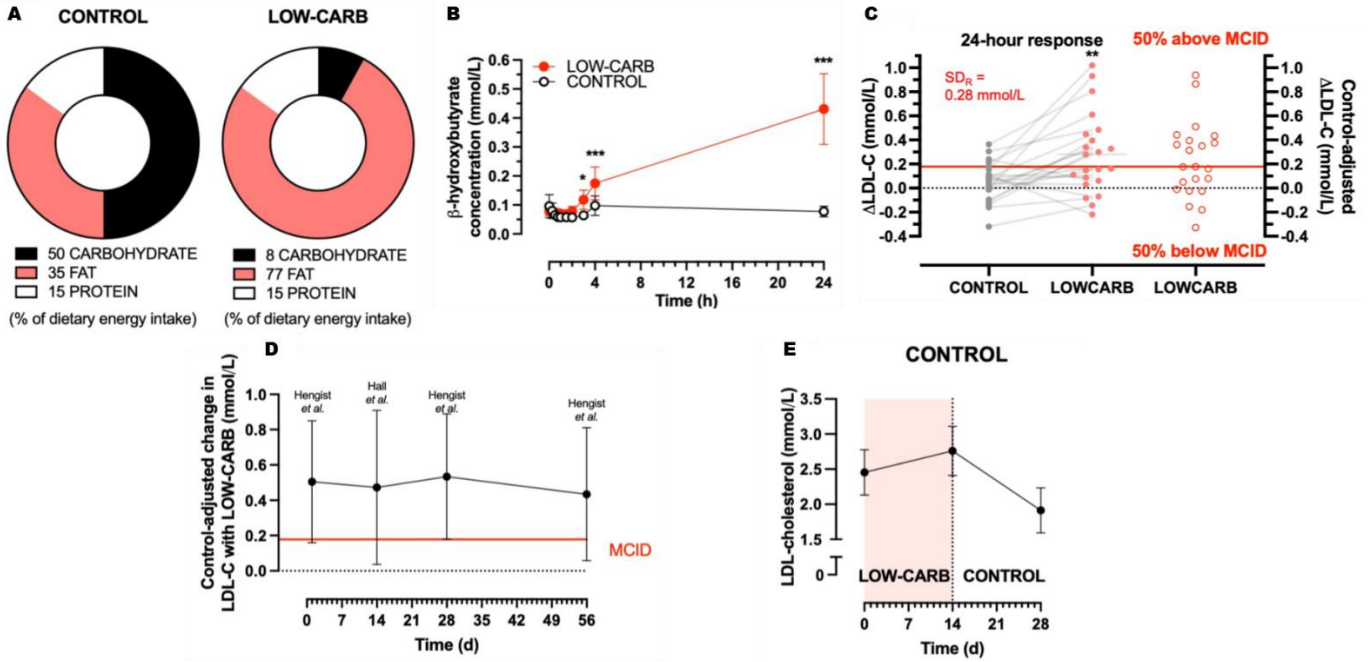

Figure-5. Twenty-five healthy adults completed 24-h of low-carbohydrate (LOW-CARB) and control (CONTROL) diets in a randomised, crossover controlled-feeding design (A). Compliance was confirmed with serum ketone body concentrations (B). The low-carbohydrate diet increased LDL-C concentrations on average within 24 hours, with individual data suggesting there may be potential for inter-individual heterogeneity of response (C). However, repeated measurements are needed to make confident inferences. These data were combined with our longer-term data (from Fig-1) plus data from Hall et a. (2021 *Nature Medicine*) to illustrate that the 24-h time course is representative of the longer-term LDL-C response (D), and also that washout of LDL-C is as rapid as the increase (E). MCID, minimal clinically important difference. *p<0.05 vs. CONTROL; **p<0.01 vs. CONTROL; ***p<0.001 vs. CONTROL.

This project will be the first to use a replicate crossover design to establish treatment-response heterogeneity to low-carbohydrate diets.

3. AIMS, OBJECTIVES AND HYPOTHESIS

AIMS: The aims of this project are to establish the degree of inter-individual heterogeneity in LDL-C and fasting glucose responses to ketogenic diets, thereby revealing the potential for personalised use of (or targeted guidance to specifically avoid) ketogenic diets for cardiovascular disease risk. Additionally, this project will establish the degree to which LDL-C and fasting glucose responses are predicted by baseline characteristics.

HYPOTHESES:

1. A replicate randomised crossover trial of a 24-hour low-carbohydrate diet reveals statistically significant and clinically relevant heterogeneity in LDL-c responses between participants.

2. The majority of participants show a significant increase in LDL-c, but these increases differ in magnitude between people.

3. A replicate randomised crossover trial of a 24-hour low-carbohydrate diet reveals statistically significant and clinically relevant heterogeneity in fasting glucose responses between participants.

4. Participants with lower biomarkers of cholesterol absorption and/or synthesis will show lower/not a significant increase in LDL-c after the low-carbohydrate diet in comparison to participants with higher numbers of these biomarkers.

5. Participants with a higher polygenic risk score (PRS) for LDL-c will show a significant increase in LDL-c after the low-carbohydrate diet in comparison to participants with lower PRS.

6. BMI will negatively correlate with the increase in LDL-c on a low-carbohydrate diet when adjusting for the control diet.

7. Participants with higher LDL receptor expression in peripheral blood mononuclear cells at baseline will show lower increases in LDL-c after the low-carbohydrate diet.

8. Changes from pre- to post-diet trial days in LDL receptor expression will be negatively correlated with changes in LDL-cholesterol.

4. PROGRAMME AND METHODOLOGY

4.1. Study Design

We will undertake a controlled feeding study with a repeated-period, randomised crossover design with 3 cycles (pairs of diet conditions), 6 x 1-day periods and 8 sequences (see below) with a 6-day washout in between each experimental period. The two diet conditions are CONTROL vs. LOWCARB. Participants will be randomly allocated to one of 8 sequences (Fig-6). The project will be conducted and reported in accordance with the CONSORT extension for reporting of n-of-1 trials statement and checklist[10].

4.2. Primary Outcome

Fasted plasma LDL-c concentrations to establish whether there is true inter-individual heterogeneity in response to 24 hours of a low-carbohydrate diet.

4.3. Secondary Outcomes

Fasted plasma glucose concentrations to establish whether there is true inter-individual heterogeneity in response to 24 hours of a low-carbohydrate diet.

4.4. Tertiary Outcomes

Other fasted metabolic health markers (low-carbohydrate vs control) helping elucidate primary outcome and potentially predictors of heterogeneity:

- NMR fatty acid profile (i.e., mono and polyunsaturated, and saturated)
- NMR amino acids (i.e., branched-chain amino acids and tyrosine)
- NMR ketone bodies (i.e., beta-hydroxybutyrate, acetoacetate, acetone)
- Plasma beta-hydroxybutyrate concentration
- Urine acetoacetate
- Plasma lactate concentration
- Plasma triglycerides concentration
- Plasma glycerol concentration
- Plasma NEFAs concentration
- Plasma insulin concentration
- Plasma C-reactive protein

Potential atherogenicity of responses to a low-carbohydrate diet (low-carbohydrate vs control):

- Plasma lipoprotein subclass profiling with lipid concentrations within 14 subclasses
- Plasma sphingomyelin- and ceramide-rich particles

Baseline potential predictor markers of LDL-c responses

- Age
- Weight
- Body Mass Index (BMI)
- Waist circumference
- Hip circumference

- Waist-to-hip ratio (WHR)
- Body composition (e.g., fat mass, fat free mass)
- Resting Metabolic Rate (RMR)
- Concentrations of biomarkers of cholesterol synthesis
- Concentrations of biomarkers of cholesterol absorption
- 12 common LDL-c raising single nucleotide polymorphisms in 11 genes
- LDL receptor mRNA content in PBMCs
- LDL receptor protein content in PBMCs

4.5. Participants and Eligibility Criteria

Examining inter-individual heterogeneity of response justifies a heterogeneous sample. This provided a starting point for the justification of inclusion/exclusion criteria, with the goal of providing a sample representative of the UK population who are healthy but spanning a wide range of body mass index (BMI). This is based on the rationale that low-carbohydrate diets are used by the general population, and whilst healthy at baseline, people may be putting themselves at unknown risk of CVD if they show a meaningful increase in LDL-C. A wide range of BMI allows the quantification of the relationship between BMI and inter-individual response to low-carbohydrate diets. This will allow us to understand whether specific individuals living with overweight and obesity may benefit from low-carbohydrate diets. Including people who are not living with overweight, and obesity allows understanding of the response across the BMI range, providing valuable context in addition to relevance for cardiometabolic health across a wider range of the population. Therefore, we will recruit a mixed of 20 participants (men and women) according to the following criteria:

<u>Inclusion Criteria:</u>

- Age: >18 years
- Body mass index: 18.5-35 $kg \cdot m^{-2}$
- LDL-cholesterol <4.9 mmol/L
- Non-fasting triglycerides <2.3 mmol/L

<u>Exclusion criteria:</u>

- weight instability (>5% change in body mass within last 3 months)
- smoker
- diagnosis of hypercholesterolaemia or any other lipid-related disease
- diagnosis of diabetes
- diagnosis of atherosclerotic cardiovascular disease
- elevated fasting glucose (>5.4 mmol/L)
- dietary intolerances or allergies or to other study procedures
- diagnosis of gastrointestinal disorders
- any other condition/medication that could introduce bias to the study
- already following a ketogenic diet
- not willing to follow a ketogenic diet
- unable to understand English language

4.6. Recruitment

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. X 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.

Diet Composition

The macronutrient composition of our diets will match our preliminary data (Table 1). The control diet macronutrient composition is reflective of typical UK intakes[13] whereas the low-carbohydrate diet is matched to the control diet for protein and for saturated-to-unsaturated fat ratio. Beta-hydroxybutyrate concentration via finger pricks and urinary acetoacetate concentrations will confirm compliance to the low-carbohydrate diet as per our preliminary data.

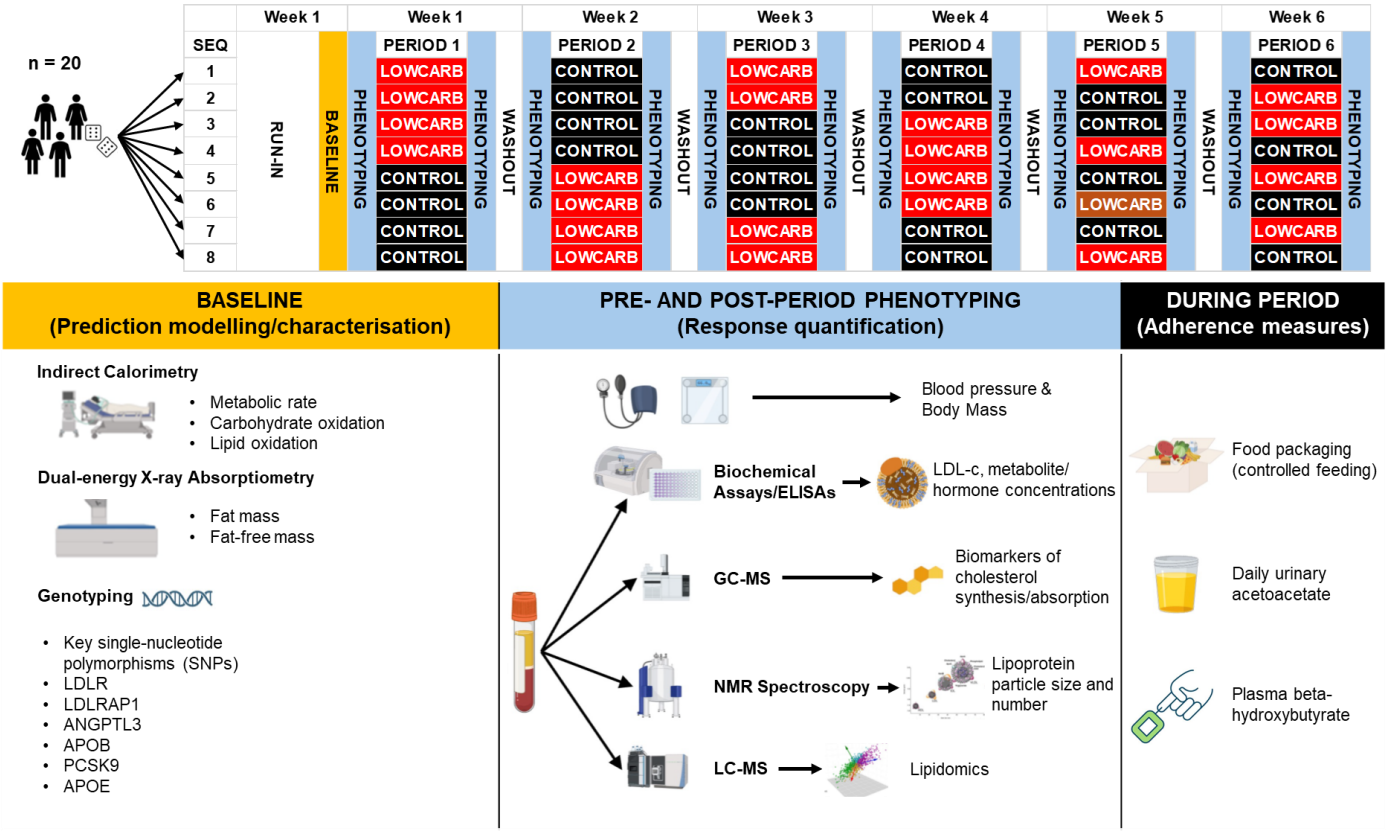

Figure-6. Summary of approach. Twenty people will be recruited and randomised to one of 8 sequences in a replicate randomised crossover study of low-carbohydrate (LOWCARB) diets. SEQ: Sequence.

Table 1. Composition of diets during controlled feeding phases – based on our pilot data.

Nutrient	CONTROL	LOWCARB
Carbohydrate (%en)	50	8
Fat (%en)	35	77
Saturated fat (%en)	14	31
Saturated to unsaturated fat ratio	0.66	0.66
Protein (%en)	15	15

%en, percentage of total energy intake; LOWCARB, low-carbohydrate.

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. pregnancy, diabetes 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 food provided.

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.

If participants are eligible, we will establish energy requirements by measuring resting metabolic rate (by indirect calorimetry), and physical activity energy expenditure (by individually calibrated heart rate and accelerometry with branched-equation modelling)[14]. A fasting blood sample will also be obtained, and genomic DNA will be isolated from the buffy coat layer for genotyping as we have previously performed[15,16].

4.8. Schedule of Measurements

Following screening, participants will follow one of the 8 sequences, randomly chosen (randomized by JG using www.randomizer.org) of the six main laboratory trial visits. The washout period between trials will be between 6 days.

Pre-laboratory visits standardisation
To assist in producing a similar individual metabolic state on the morning of laboratory trial days, participants will be asked to record physical activity for 24 h before first trial and during diet day and replicate for the next ones.

Laboratory trial days (morning of diet and next morning)
Participants will arrive at the laboratory in a rested, overnight fasted state, after 24-hours of a low-carbohydrate or control diets.

In each trial day body mass, height, waist and hip circumference will be recorded. Participants will then rest 15 minutes before blood pressure can be recorded. Venepuncture will be performed in an antecubital vein and 20 ml of blood will be obtained before and after each diet period to determine concentrations of LDL-C and glucose in addition to other lipoprotein characteristics and metabolite/hormone concentrations to provide further information on cardiometabolic health and predictive biomarkers.

Replicate RCT Ketogenic Diet Protocol
On the first laboratory day a dual-energy x-ray absorptiometry (DEXA) scan will be performed to calculate body composition, and for potential additional predictive biomarkers.

Lastly, for exploratory analysis in future work, we will also collect urine samples before and after each diet day.

Diet
After participants have gone through the laboratory measurements, the meals for the diet to be followed for 24 hours will be provided by the research team. Participants will be asked to consume only what has been given to them. The total calories will be based on the calculated metabolic rate done during the screening.

4.9. Measurements and Analysis

Metabolite and hormone concentrations
Plasma LDL-cholesterol, glucose, beta-hydroxybutyrate, lactate, TG, glycerol, non-esterified fatty acids, HbA1C (baseline only) and insulin and C-reactive protein concentrations will be determined by colourimetric or enzyme-linked immunosorbent assays as is routine in our laboratory[11,15,17]. Handheld devices will also be utilised as a backup to analyse blood glucose, lactate and beta-hydrobutyrate. These will be used to provide the primary outcome measure, in addition to other important contextual information on the broad metabolic response to the diets. In addition, these will also provide information on potential predictors of response heterogeneity. We will also measure lipoprotein(a) concentrations as the most atherogenic lipoprotein in a per particle basis and potentially responsive to diet[18,19].

Biomarkers of cholesterol synthesis and absorption
Plasma samples will also be analysed by gas chromatography-mass spectrometry (GC-MS) to determine concentrations of biomarkers of cholesterol synthesis (e.g., 7-dehydrocholesterol, desmosterol and lathosterol) and of cholesterol absorption (e.g., sitosterol and campesterol). This will establish if these biomarkers are useful as predictive factors for the heterogeneity of the LDL-C response to a low-carbohydrate diet.

LDL receptor expression
The expression of the LDL-receptor (LDL-R) in the liver could explain response heterogeneity[20]. Dietary fat content and quality (saturated to unsaturated ratio) affects hepatic LDL-R clearance of LDL in rodents via reduced hepatic LDL-R mRNA and protein expression[20,21]. As a human study in healthy participants, sampling liver tissue would be technically and ethically challenging. However, hepatic LDL-R mRNA content has been shown to directly correlate with hepatic LDL-R protein content in rodents[21], and LDL-R mRNA in human liver directly correlates with LDL-R mRNA in peripheral blood mononuclear cells ($r=0.9$, $p<0.001$)[22]. Changes in LDL-R also directly correlate with changes in circulating lipoproteins in response to fat intake[23]. Therefore, the LDL-R expression of PBMCs reflects hepatic LDL-R protein content. We will isolate PBMCs via density gradient centrifugation and quantify LDL-R mRNA and protein content of PBMCs via reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting, respectively, to establish the role of LDL-R expression in mediating the effects of low-carbohydrate diets on LDL-C concentrations.

Nuclear Magnetic Resonance (NMR) spectroscopy
Plasma samples will also be analysed by high-throughput NMR (Nightingale Health Plc) which quantifies >150 metabolic biomarkers, including routine lipids, lipoprotein subclass profiling with lipid concentrations within 14 subclasses, fatty acid composition, and various low-molecular-weight metabolites such as amino acids, ketone bodies, and glycolysis metabolites. This panel provides information on lipoprotein particle concentrations and characteristics (e.g., diameter, lipid content

and composition), which provides more understanding of atherogenicity over concentrations alone. This will also provide information on metabolite concentrations to further explain the heterogeneity in fasting glucose concentrations and add to overall cardiometabolic health profile (e.g., branched-chain amino acids). Furthermore, this will allow for linkage with the UK biobank for additional exploratory analyses.

Lipidomics (subject to future funding)

Since all LDL particles are potentially atherogenic, LDL particle number is a primary determinant of atherogenesis[24]. However, additional characteristics also play a role in the full atherogenic potential of LDL particles via modulating susceptibility to aggregation, oxidation or proteoglycan binding[24]. Sphyingomyelin- and ceramide-rich particles are often more aggregation-prone than phosphatidylcholine-and lysophatidylcholine-rich particles[25]. To further explore the potential atherogenicity of responses to low-carbohydrate diets, we will perform quantitative lipidomics on plasma samples via liquid chromatography mass spectrometry (LC-MS).

Genotyping

We will genotype participants for 12 common LDL-C-raising single nucleotide polymorphisms (SNPs) in 11 genes (two in APOE) which have been identified as causing polygenic elevated LDL-C in UK participants of several ethnicities (Table 2[26,27]). DNA extraction and genotyping will be performed in line with our previous publications using commercially available extraction kits (Qiagen) and Taqman probes[15,16].

Table 2. LDL-C raising single nucleotide polymorphisms

SNP	Gene	Chromosome number	Start position (GRCh37)	Allele		UK allele frequencies		
				Common	Minor	W	B&C	SA
rs2479409	PCSK9	1	55504650	G	A	0.6516	0.7484	0.7481
rs629301	CELSR2	1	109818306	G	T	0.7777	0.6297	0.7362
rs1367117	APOB	2	21263900	G	A	0.3388	0.0856	0.1584
rs4299376	ABCG8	2	44072576	G	T	0.6766	0.8469	0.7413
rs1564348	SLC22A1	6	160578860	T	C	0.1705	0.1078	0.1486
rs1800562	HFE	6	26093141	G	A	0.0777	0.0055	0.0022
rs3757354	MYLIP	6	16127407	C	T	0.2022	0.3700	0.3020
rs11220462	ST3GAL4	11	126243952	G	A	0.1325	0.0258	0.1373
rs8017377	NYNRIN	14	24883887	G	A	0.4748	0.1338	0.3938
rs6511720	LDLR	19	11202306	G	T	0.1189	0.1423	0.0779
rs429358	APOE*	19	45411941	T	C	0.1563	0.2298	0.0967
rs7412	APOE*	19	45412079	C	T	0.0810	0.1130	0.0442

Body mass and blood pressure

In the overnight fasted-state body mass will be measured using balance scales with minimal clothing and after voiding. Body composition will be determined by dual-energy x-ray absorptiometry. Blood pressure will also be determined in triplicate using an automated sphygmomanometer.

4.10. Sample Size

Sample size justification will follow a pragmatic approach[28], with planning and research design considerations based on k=2 per participant minimum number of cycles required for a replicated crossover research design[32]. For detection of treatment response heterogeneity, a moderate-to-high and positive between-replicate correlation coefficient (r) is an important indicator of stable or 'true' within-person treatment responses[9,29-32]. A sample size of 20 would enable an *r* as low as 0.38 [90%CI: 0.00-0.66] to be detected as statistically significant (one-tailed P=0.05). In terms of mean treatment detection, our pilot data (*n*=22, Fig-4), indicate that acute mean ± SD treatment effects

were 0.22±0.29 mmol/L (*P*=0.002) for LDL-C. It is estimated that statistical power to detect these effect sizes would be further improved to over 99% because of the repeated administration of conditions (reducing within-subject random variance and local trend effects[31,32]).

4.11. Statistical Analyses

Statistical analyses will be undertaken in a blinded fashion. Data will be reported in accordance with CONSORT extension for n-of-1 trials (CENT) guidelines[10]. Standard residual diagnostics will be undertaken to assess the influence of any violated assumptions on the adequacy and the stability of the modelled covariance parameter estimates[34,35].

Primary, Secondary and Tertiary analyses
According to Senn[9,31] data will be analysed with a within-subjects linear mixed model (see Senn[32] for typical SAS code). Using the PROC MIXED procedure in SAS OnDemand for Academics (SAS Institute), the within-participant linear mixed model and repeated design will allow quantification of any participant-by-condition interaction for each outcome. Condition and period (sequence), will be modelled as fixed effects, and participant and participant-by-condition terms will be modelled as random effects. These analyses will be supplemented by the between-replicate correlation approach[32] and, most recently, the meta-analytic approach detailed by Senn[33], which has been used before in the analysis of reactive hypoglycaemia after breakfast[8].

Quaternary analyses
Will be explored by including potential predictive biomarkers in the model as fixed between-subjects effects and the biomarker-by-condition interaction will be quantified[16]. Multi-variable linear regression will be used to produce a prediction model.
To produce an efficient exploratory prediction model, NMR spectroscopy and lipidomics outcomes will first undergo a screening process to select inputs for prediction modelling. This process will involve examination of Pearson's product-moment correlations between each potential biomarker and the replicated CONTROL-adjusted LDL-C response to LOW-CARB. The 5 outcomes with the largest correlation with the replicated, CONTROL-adjusted response of LDL-C to LOW-CARB will be taken forward into the prediction model along with genotype and biomarkers of cholesterol synthesis and absorption. Finally, to better understand the association between SNPs and LDL-C response a variety of statistical approaches will be explored.

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.

All data will be managed, stored, and curated at the University of Bath. Newly generated data will be stored on university managed servers and on the university Research Data Archive. The Data Steward (Javier Gonzalez) initiates a repository and limits access permissions to only include authorised researchers. Scripts relating to dataset generation are stored securely in the same location as the dataset and will be shared in a public online repository (GitHub) upon manuscript publication (with information including specific file paths removed). Research data used in analyses are structured and version-controlled in accordance with Standard Operating Procedures, whereby when a dataset is ready for use in analyses, or published, a serialized snapshot following peer-review will be generated and placed in the released area of the repository.

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 be provided with an information pack containing their results for relevant outcomes along with reference ranges (e.g., 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 through a venepuncture, 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.

Participants will be asked to also provide urine samples, and although there is little health/contamination risk, there could exist some discomfort from participants. Therefore, if that is the case, we will not require those samples from participants.

There is a risk of radiation exposure with the DEXA scan although the amount of radiation is extremely low. The radiation dose for one DEXA is less than one day of background radiation and is less than the amount of radiation used for a diagnostic x-ray. Throughout the study, participants will undergo 1 whole-body DEXA scan. In total this will provide a radiation dose of ~0.01 mSv (Blake et al., 2006). Pregnant and other contraindicated populations will be excluded from the study. A radiologist and medical physics expert will oversee our ethics application (as standard).

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.

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