Study Acronym	**VitaDEx**
Study Title	**Mobilising vitamin D sequestered in adipose tissue in humans with Exercise**

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Sponsor:	University of Bath
Funder:	BBSRC

CONTENTS

1 SYNOPSIS

Study Title	**Mobilising vitamin D sequestered in adipose tissue in humans with Exercise**
Study Acronym	VitaDEx
Trial Design	Randomised Controlled Trial in overweight men and women (with a lean referent group)
Trial Participants	Men and women aged 25-65
Intervention	Overweight: 10-week exercise intervention or control Lean: None
Planned Sample Size	70 (50 overweight, 20 lean)
Primary Objective	To establish whether exercise improves serum vitamin D status in men and women and to investigate whether this translates into improved biological action of vitamin D
Secondary Objectives	To characterise the impact of exercise on vitamin D turnover and metabolism To determine whether exercise improves the ability to mobilise vitamin D from adipose tissue in basal conditions and in response to stimulation (*ex vivo* and *in vivo*)
Primary Outcomes	The effect of structured exercise on vitamin D 'status' (serum 25OHD)
Secondary Outcomes	The effect of structured exercise AND adiposity on: • Serum concentration of other vitamin D metabolites and vitamin D binding proteins • Urinary excretion of vitamin D metabolites • Whole-body vitamin D turnover and metabolism (using stable isotopes) • Vitamin D bioavailability • Adipose and skeletal muscle vitamin D content • Adipose vitamin D mobilisation (ex vivo) • Adipose tissue transcriptome (RNAseq) with targeted protein measurements (Western blotting) • Adipose and muscle arteriovenous differences for vitamin D metabolites in response to stimulation (in a subset of participants only) • Adipose and skeletal muscle acylcarnitine metabolism • Serum metabolome, lipidome and adipokines • Fasted (and fed) serum hormones (e.g., insulin and appetite regulatory hormones) • Blood and adipose tissue immune cell phenotype (flow cytometry) • Virus-specific antibody status (Cytomegalovirus and Epstein-Barr virus) • Incidence of upper-respiratory tract infections (URTI's) • Gut microbiome taxonomic composition and metabolic potential
Funder	BBSRC
Sponsor	The University of Bath
Principal Investigator	Dr Oliver Perkin
Chief Investigator	Professor Dylan Thompson

2 BACKGROUND AND RATIONALE

Executive Summary

Sequestration of lipophilic vitamin D in adipose tissue creates a challenge to the maintenance of circulating (serum) vitamin D. As the prevalence of overweight and obesity increases, it is important to find successful strategies to mobilise vitamin D from adipose tissue and thus improve the bioavailability of endogenous vitamin D.

Our preliminary data indicate that physical activity is an effective way to mobilise vitamin D from adipose tissue – even without weight loss. These preliminary data show that exercise has a powerful effect on the concentration of the important form of vitamin D in blood and that exercise stimulates the release of this form of vitamin D from adipose tissue. Based on these observations, we hypothesise that regular exercise will improve the mobilisation of vitamin D from adipose tissue and that this will increase the amount and availability of the important form of vitamin D in blood.

To test this hypothesis, we will use a 10-week randomised controlled trial in overweight men and women to examine the impact of exercise (versus control) on vitamin D status and metabolism. We will assess the impact of exercise on the various forms of vitamin D that are found in blood, and whether these changes lead to an improvement in the function of cells known to be affected by vitamin D status (a type of white blood cell called monocytes). We will use stable isotopes (non-radioactive tracers) to examine how the turnover of vitamin D is affected by exercise. By taking small biopsies of adipose tissue we will be able to examine the impact of exercise on the ability to mobilise vitamin D from adipose. This will also allow us to understand the biological pathways and mechanisms that are involved in vitamin D mobilisation and how they are affected by exercise. We have designed our research to include a lean comparator group so that we can contextualise the effects of the intervention and understand the independent effects of adiposity on vitamin D mobilisation, status and metabolism.

Relevant literature

Vitamin D and health

Vitamin D has effects far beyond its classical actions on calcium homeostasis and bone metabolism, and a low level of vitamin D affects many physiological systems and has been linked to a wide array of human health problems [3-5]. In recognition of the importance of vitamin D for health, the UK government issues policy statements and guidance regarding vitamin D requirements via Public Health England. Vitamin D status is conventionally determined by measuring concentrations of the main circulating form of vitamin D, 25-hydroxyvitamin D (25OHD). Approximately 25-50% of the UK population is vitamin D deficient [6] and the correction of low systemic concentrations of 25OHD is a recognised public health priority [7, 8].

Adipose tissue 'traps' vitamin D

Overweight and obesity is associated with vitamin D deficiency [9] due in part to sequestration of lipophilic vitamin D (primarily cholecalciferol/vitamin D_3) within lipid droplets of adipose tissue [10-16]. Thus, poor vitamin D status in obesity is not solely due to low whole-body vitamin D content but, instead, often reflects the fact that a significant proportion of total vitamin D is 'trapped' within adipose tissue. This entrapment is partly due to the increased capacity to sequester vitamin D in this enlarged compartment (adipose masses are increased by 20-40 kg in modest class I obesity and adipose accumulates up to ~120 ng vitamin D_3 per g adipose even without supplementation [15]). On top of an expanded capacity for sequestration, adipocyte function becomes impaired with obesity and there is resistance to physiological stimulation, manifested as a reduced capacity to mobilise both lipid [17, 18] and vitamin D [16]. Thus, vitamin D accumulation in overweight/obese adipose tissue is likely a function of both the enlarged capacity to sequester vitamin D and impaired mobilisation of vitamin D from dysfunctional adipocytes. The issue of whether adipose acts as a store or non-reversible sink for vitamin D and its metabolites was one of 12 main recommendations for future research in the recent UK Government Scientific Advisory Committee on Nutrition (SACN) report on vitamin D and health ([19] p141).

Strategies to release 'trapped' vitamin D from adipose

There is reasonable evidence that substantial weight loss increases circulating 25OHD [20-23] and improvements are associated with total adipose tissue loss [24]. The likely explanation is that vitamin D metabolites are released in tandem with fatty acids mobilised from adipose in response to the energy deficit. Consequently, it has recently been suggested that treatment of vitamin D deficiency should emphasise the importance of reductions in adipose tissue mass [25]. Long-term weight loss is challenging and difficult. We propose that weight loss is not the only way to mobilise vitamin D metabolites trapped within adipose tissue – and that physical activity (even without weight loss) may represent another highly successful strategy for improving systemic concentrations of 25OHD.

Physical activity has pronounced effects on adipose tissue

Physical activity is a very powerful stimulus for lipid mobilisation from adipose tissue [17]. Rates of lipolysis during and after even low intensity physical activity such as walking are 3 to 5-fold above rest and much higher than observed during extreme calorie restriction [26, 27]. We postulate that physical activity-induced lipid mobilisation from adipose tissue will also promote the release of lipophilic vitamin D sequestered within adipocytes. Furthermore, it is well established that an increase in physical activity will chronically improve adipocyte function even without weight loss [17]. Exercise training increases sensitivity to various mediators such as insulin and adrenaline in overweight/obese adipose tissue [17]. Thus, in addition to an acute effect on 25OHD release associated with each exercise bout, we also anticipate that regular exercise will improve adipocyte function and the capacity to respond when stimulated; including the capacity to mobilise 25OHD in response to habitual physical activity, fasting and stress. In support of our proposition, large observational studies have reported serum 25OHD to be significantly higher in physically active people [28]. Such associations have often been explained-away by the notion that active people might spend more time outside, but this is largely supposition. Instead, we hypothesise that physical activity impacts positively on vitamin D status because it causes the mobilisation of vitamin D metabolites from adipose tissue.

Preliminary Data

Exercise interventions increase circulating 25OHD

To provide preliminary support for our hypothesis that physical activity improves vitamin D status, we assessed the impact of exercise on circulating concentrations of 25OHD in humans (Figure 1A). These findings are corroborated by data in an animal model from an independent group (Figure 1B). Collectively, these preliminary data from two independent intervention studies show that increased physical activity – without weight loss – can cause an increase in circulating concentrations of 25OHD by ~30% relative to time-matched controls:

Figure 1A: We provide the first causal evidence that physical activity increases 25OHD in humans relative to time-matched controls (i.e., increased physical activity ameliorates the winter decline). These data come from a secondary analysis of blood samples from an exercise RCT in overweight middle-aged men completing 24 weeks of exercise or control (no exercise) [29]. We analysed samples from participants who were enrolled at the end of summer and undertook the intervention in the winter months when there would be little new vitamin D synthesis in the UK. In the control group, 25OHD fell by ~40% from summer to winter, whereas in the exercise group the fall was only ~10%. The supervised exercise was conducted indoors and thus this effect is not due to exercisers spending more time outside. There was very little exercise-induced weight loss; thus, exercise *per se* offset the typical decrease in 25OHD experienced through the winter months.

Figure 1B: Data from an independent team show that physical activity mobilises endogenous 25OHD in an animal model. This study found that 8 days of daily exercise in dogs increased 25OHD by ~30% [30]. Dietary intake of vitamin D was controlled,

synthesis of 25OHD was low because of the dogs' thick fur coats and the control animals were also kept in the same conditions. There was also no weight loss; thus, the increase in 25OHD in these animals was due to the mobilisation of endogenous vitamin D stores with exercise.

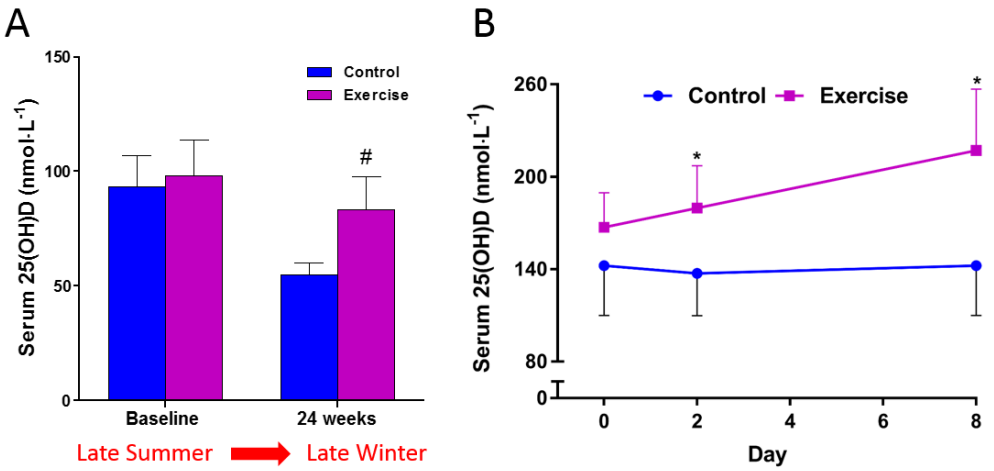

Figure 1. *Serum 25OHD concentrations* ***(A)*** *in exercise versus non-exercising humans across a 24-week exercise intervention based on analysis of samples through the winter months from [29] (n=6 & 5 for EX and CON, respectively; # Effect Size = 0.95 (ES = M_2-M_1/SD pooled, >0.8 equals a large ES); and* ***(B)*** *in sled dogs in response to consecutive days of exercise versus matched controls [30] (n=12, * ES = 2.0, p<0.05).*

25OHD is released from adipose tissue explants after exercise

To build on these whole-body observations, we undertook further preliminary *ex vivo* work to establish whether an acute bout of exercise causes adipose tissue to release 25OHD. We analysed media from adipose explants after overweight/obese participants exercised at 60% VO_2 max for 60 minutes (Figure 2). Adipose biopsies were obtained at baseline and 1 h post-exercise and explants were cultured for 3 h as previously described [1] [2]. Exercise had a large effect on release of 25OHD from adipose tissue (Figure 2). These data demonstrate that human adipose tissue releases 25OHD in the hours after exercise and this provides one mechanism for the impact of regular exercise on systemic 25OHD shown in Figure 1.

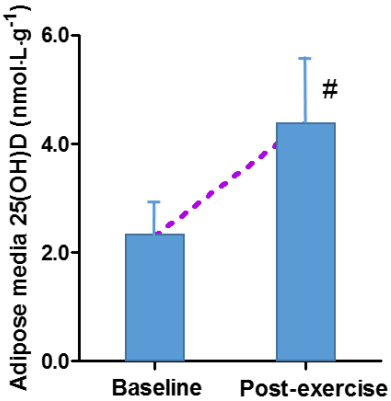

Figure 2*: Net release of 25OHD from human adipose tissue before and 1h after 60 min of moderate intensity exercise (n=5). Adipose explants were cultured for 3h as described previously [1] [2].* ***#*** *Exercise had a large effect on 25OHD release (ES= 0.96). These results show that adipose continues to release 25OHD for some hours after exercise. All participants were overweight but with varying degrees of adiposity (total fat mass ranged from 24-45 kg).*

Timeliness of this research

This project addresses an important research gap. The issue of whether adipose acts as a store or non-reversible sink for vitamin D and its metabolites was one of 12 main recommendations from the UK Government Scientific Advisory Committee on Nutrition (SACN) for future research ([19] p141). This recent report on vitamin D and health highlighted that "*the extent to which the processes of accumulation and mobilisation* [of vitamin D] *are regulated by normal physiological mechanisms remains unknown*" (p28) and that "*details about accumulation and mobilisation of vitamin D from adipose tissue and other tissues are not clear at the present time*" (p131) [19]. The current project will establish whether increasing physical activity mobilises vitamin D from adipose tissue. This has important ramifications for practitioners and policy makers regarding the management of (i) low vitamin D, (ii) obesity and associated conditions and (iii) low levels of physical activity and/or immobilisation/bed rest. These are major health priorities.

Novelty and relevance of this research

Current public health strategies typically approach vitamin D deficiency by increasing intake and/or synthesis of vitamin D (e.g., dietary supplementation or UV treatment). Notably, overweight/obesity reduces the impact of dietary supplementation with vitamin D on 25OHD [31-34] and the systemic 25OHD response to ultraviolet (UV) irradiation is significantly impaired [11]. Thus, complementing conventional intake/synthesis strategies with techniques to mobilise endogenous vitamin D is a novel approach that will mutually enhance the overall effectiveness of interventions to improve vitamin D status. This paradigm also opens up the intriguing possibility that physical activity might release other lipophilic molecules 'trapped' in adipose tissue (e.g., lycopene, vitamin E etc.).

3 OBJECTIVES

Our preliminary data strongly indicate that increased physical activity improves circulating 25OHD relative to time-matched controls and that a single bout of exercise increases the release of 25OHD from adipose tissue for at least several hours. These novel observations provoke many inter-related unanswered questions. How does the mobilisation of vitamin D from adipose tissue affect overall turnover, metabolism and excretion? What is the effect of exercise on other vitamin D metabolites? Does the change in systemic 25OHD make a difference to biological function? What proportion of adipose vitamin D is mobilised? Is the effect of regular exercise due to enhanced basal mobilisation associated with improved adipocyte function? Or, is the effect mostly driven by acute mobilisation in parallel with enhanced lipolysis during each exercise bout? Thus, to answer these questions we have established the following objectives for the current study:

Primary Objectives

1. To establish whether exercise improves serum vitamin D status in men and women and to investigate whether this translates into improved biological action of vitamin D

2. To characterise the impact of exercise on vitamin D turnover and metabolism

3. To determine whether exercise improves the ability to mobilise vitamin D from adipose tissue in basal conditions and in response to stimulation (*ex vivo* and *in vivo*)

Experimental Hypothesis

Regular exercise will improve the mobilisation of vitamin D metabolites from expanded adipose tissue and thus improve serum vitamin D status and bioavailability.

4 PROGRAMME AND METHODOLOGY

Study Design

This study has two parts. The main part of the study will be a 10-week RCT in overweight men and women (exercise *versus* control). This RCT will address the primary objectives and determine the impact of structured exercise on vitamin D metabolism. In addition to the RCT, and in order to adequately contextualise the effects of exercise and enable an assessment of the extent of 'normalisation' of adipose tissue mobilisation of 25OHD, we will also recruit an age-matched active lean group. In addition to contextualising the effects of the intervention, an overweight *versus* lean comparison will characterise the effects of adiposity *per se*.

In the RCT, men and women will be allocated to an exercise intervention or control group (n=50; 25:25). The comparator group will be age-matched active lean men and women (n=20). The comparator group will not undertake any intervention.

Variability in UV-induced vitamin D synthesis is a potential confounder and therefore we will structure the experimental work to minimise the impact of UV exposure. Thus, whilst recruitment will take place over most of the year, we aim to conduct the experimental work during the winter months when there is little vitamin D synthesis at this latitude [35]. Most testing will take place between October and March but some participants may be tested in September and April (due to scheduling). Participants who take part in these months will be provided with guidance to wear sunscreen if they are going outside and to wear a hat and clothes with long sleeves.

Primary Outcome

The effect of structured exercise on vitamin D 'status' (serum 25OHD)

Secondary Outcomes

The effect of structured exercise AND adiposity on:

- Serum concentration of other vitamin D metabolites and vitamin D binding proteins
- Urinary excretion of vitamin D metabolites
- Whole-body vitamin D turnover and metabolism (using stable isotopes)
- Vitamin D bioavailability
- Adipose and skeletal muscle vitamin D content
- Adipose vitamin D mobilisation (ex vivo)
- Adipose tissue transcriptome (RNAseq) with targeted protein measurements (Western blotting)
- Adipose and muscle arteriovenous differences for vitamin D metabolites and other metabolites in response to stimulation (in a subset of participants only)
- Serum metabolome, lipidome and adipokines
- Fasted (and fed) serum hormones (e.g., insulin and appetite regulatory hormones)

- Blood and adipose tissue immune cell phenotype (flow cytometry)
- Virus-specific antibody status (Cytomegalovirus and Epstein-Barr virus)
- Incidence of upper-respiratory tract infections (URTI's)
- Gut microbiome taxonomic composition and metabolic potential

Eligibility Criteria

10-week RCT in overweight men and women

Inclusion Criteria:

- Aged 25-65 years
- Fat Mass Index (FMI) determined using DEXA of 7.5-15 kg/m^2 (♂) and 11-21 kg/m^2 (♀).
- Self-reported participation in no vigorous intensity physical activity and less than 150 minutes of moderate intensity activity per week AND an objectively assessed Physical Activity Level (PAL) less than 2.00 (total energy expenditure/resting energy expenditure)
- Available during the winter months (October-March)
- Non-smoker

Exclusion Criteria:

- Current or recent use of weight loss drugs
- Any reported recent (i.e. last 6 months) shift (>5%) in body mass or large change in habitual lifestyle.
- Individuals with coronary heart disease, chronic kidney disease, type 2 diabetes, stroke, heart failure and peripheral arterial disease.
- Individuals with 'severe hypertension' defined as a blood pressure greater than 180/110 mmHg (British Hypertension Society and NICE guidelines – CG127).
- Individuals unable to change their physical activity (e.g. through disability).
- Positive responses to the Physical Activity Readiness Questionnaire (PAR-Q)
- Recent (i.e. last 2 months) participation in another research trial or lifestyle supportive intervention. (Participants will be asked not to donate blood whilst participating in the study)
- Taking medication that might interfere with the study outcomes
- Regular consumption of dietary supplements containing vitamin D within the last three months
- Regular use of sunbeds within the last three months
- Abnormal resting ECG (for arteriovenous difference participants, only)
- Sensitivity or allergy to lidocaine or any local anaesthetic medicines.
- Pregnancy

Lean Comparator Group

Inclusion Criteria

- Aged 25-65 years
- Fat Mass Index (FMI) determined using DEXA of 2-6 kg/m^2 (♂) and 4-9 kg/m^2 (♀).
- Self-reported participation in either 150 minutes of moderate intensity or 75 minutes of vigorous intensity physical activity per week AND an objectively assessed Physical Activity Level (PAL) greater than 1.75
- Available during the winter months (October-March)
- Non-smoker

Exclusion Criteria

- Current or recent use of weight loss drugs
- Any reported recent (i.e. last 6 months) shift (>5%) in body mass or large change in habitual lifestyle.
- Individuals with coronary heart disease, chronic kidney disease, type 2 diabetes, stroke, heart failure and peripheral arterial disease.
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- Sensitivity or allergy to lidocaine or any local anaesthetic medicines.
- Pregnancy

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.

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. smoking status, use of sunbeds, pregnancy etc.) and some basic demographic information for participant characterisation (menopausal status), and other practical screening questions (e.g. any reason why participants could not exercise for 10 weeks during winter months, etc.) will be self-reported by the participant via a health screen questionnaire. Lidocaine allergy will also be assessed in this questionnaire, and a Patient Specific Directive (a prescription) will be signed by a registered clinician before any lidocaine is administered. Food allergies will be reported in this questionnaire to ensure that standardised meals for the study are suitable, and potential alternatives can be considered.

Skin colour: Participants skin colour will be assessed using the Fitzpatrick Skin Phototype questionnaire, with participants skin colour classed as type I – VI based on scores (0-40) on the 10 question scale.

Physical activity: The international physical activity questionnaire (IPAQ) short-form is validated to assess moderate and vigorous intensity physical activity and sedentary

time [36]. Participants will be asked whether their responses are representative of the past year (i.e., whether their physical activity level has been stable).

Blood pressure: An automated sphygmomanometer will be applied to the left arm following 25 minutes of rest. Systolic and diastolic pressure (mmHg) and pulse (bpm) will be measured three times in a seated position with 1 minute between each measurement.

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 (DEXA): The participant will be positioned supine in a dual-energy x-ray absorptiometry (DEXA) 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.

Physical activity monitoring: At the end of this visit, the participant will be given an Actiheart physical activity monitor (Cambridge Neurotechnology Ltd). This is attached to the chest either using ECG pads or a chest strap and will be worn for 9 days (to capture 7 full days) for measurement of free-living physical activity [37]. Data from complete days (>80% wear time) will be used to determine habitual physical activity (including PAL and time engaged in moderate/vigorous intensity physical activity). To calibrate the Actiheart, participants will be asked to perform a short submaximal walk on a treadmill with heart rate recorded.

Schedule of Measurements

Once participants have completed screening and have been randomised (if relevant) a date to take part in the study will be agreed and scheduled during the winter months (i.e., October-March). Measurements will be taken before, during and after the exercise intervention or control (overweight/obese) or on one occasion only (lean comparator) as indicated in Figure 3. Participants will be asked at screening if they would like their GP's to be informed of their participation, and if they consent to incidental findings (bone mineral density and ECG results only) being reported to their GP, by way of a pre-approved letter from the research team.

Aftercare: In the schedule described below, on any occasion when participants are asked to arrive to the laboratory in the overnight fasted state and either provide a blood sample or undertake exercise, they will then be provided with a meal and drink of their choice.

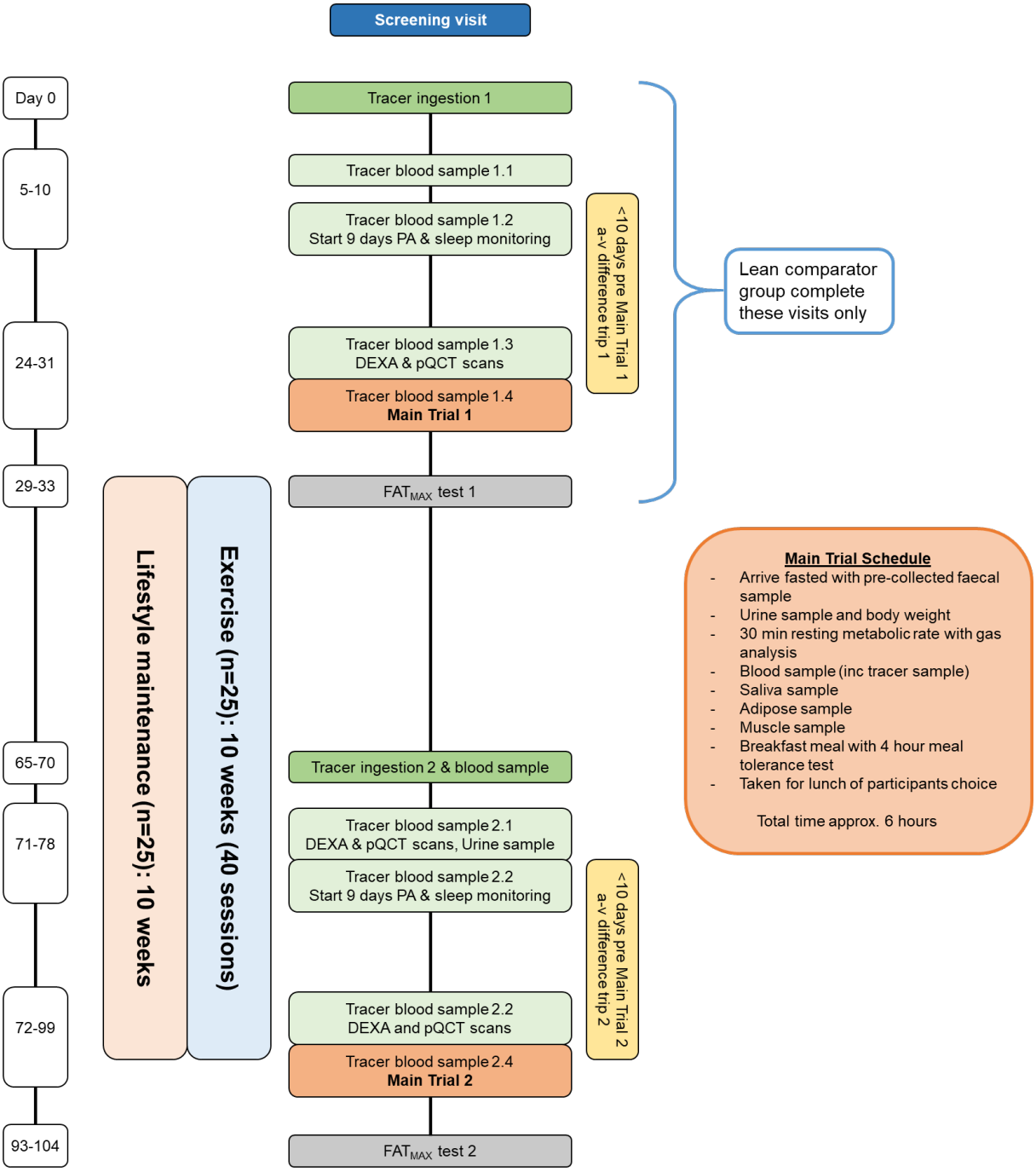

Figure 3: Overall study design and schedule of measurements

Schedule: ALL PARTICIPANTS

Visit 1 (day 0)

After an overnight fast, participants will be weighed, and will consume the stable isotope of 25OHD tracer as part of a breakfast meal. This can take place somewhere at the participants convenience.

Visit 2 (day 5-7)

Participants will be asked to attend the human physiology laboratories at the University of Bath to be weighed, and for the collection of a 3-hour fasted venous blood sample for the tracer measurement

Visit 3 (day 6-10)

Participants will be weighed, and a 3-hour fasted venous blood sample will be collected for the tracer measurement (~30 minutes). At the end of this visit, participants will be given an Actiheart physical activity monitor and a sleep monitor to wear for 9 days. They will be asked to weigh their food and fluid intake over 3 days. This visit can take place somewhere at the participants convenience.

Visit 4 (day 24-28)

Participants will be asked to attend the human physiology laboratories to be weighed, and for the collection of a fasted (3 hours since last meal) venous blood sample for the tracer measurement. We will also assess body composition using DEXA, and bone and muscle properties of the shank using peripheral quantitative computed tomography (pQCT) (~45 minutes).

Visit 5 (day 27-31)

Participants will report to the laboratory in the morning following a 10h fast. Participants will be asked to arrive with a faecal sample. They will be asked to void their bladder and provide a urine sample, and body mass will be measured. Subsequently, Resting Metabolic Rate (RMR) will be assessed by collecting expired air for thirty minutes while the participant rests lying on a bed. A baseline blood sample (~20 ml) will then be collected via a cannula. A saliva sample will also be collected. Subsequently, an adipose sample will be collected from the area around the waist, approximately 5cm lateral to the umbilicus. A muscle biopsy sample will be then obtained from the vastus lateralis. After these measurements, participants will commence a Mixed Meal Tolerance Test (MMTT). Small cannula blood samples will be collected at 15, 30, 45 minutes, 60 minutes and then every 30 minutes for 4 hours. Expired gas samples will be collected during the MMTT. Participants will be taken for lunch after the end of the MMTT.

Participants will be asked to provide a faecal and urine sample on the trial day using a provided kit. These samples must be collected no earlier than the night before and kept on ice if collected before arriving at the laboratory.

Visit 6 (day 29-33)

Participants will report to the laboratory in the fasted state to undertake a FAT_{MAX} treadmill test. For participants in the RCT Exercise group, this will be the first exercise session, and subsequent training intensities set based on this data.

Schedule: RCT PARTICIPANTS ONLY

Visit 7 (day 65-70)
After an overnight fast, a 5 mL blood sample will be taken, and participants will consume the stable isotope of 25OHD tracer (30 mins). This can take place somewhere at the participants convenience.

Visit 8 (day 71-78)
Participants will be asked to attend the human physiology laboratories for the collection of a 3-hour fasted venous blood sample for the tracer measurement. Participants will be asked to provide a urine sample. A saliva samples will also be collected. We will also assess body composition using DEXA, and bone and muscle properties of the shank using pQCT, and ask participants to perform a walk on the treadmill for Actiheart calibration ahead of their next physical activity monitoring period (~60 minutes).

Visit 9 (day 72-81)
A 3-hour fasted venous blood sample will be collected for the tracer measurement (~30 minutes). At the end of this visit, participants will be given an Actiheart physical activity monitor to wear for 9 days, and a 7-day sleep diary. They will be asked to weigh their food and fluid intake over 3 days. This visit can take place somewhere at the participants convenience.

Visit 10 (day 90-99)
Following an overnight fast, participants will be asked to attend the human physiology laboratories for the collection of a venous blood sample for the tracer measurement, and DEXA and pQCT scans (~45 minutes).

Visit 11 (day 91-102)
This visit will be a repeat of the first main trial i.e. visit 5 (~6 hours).

Visit 12 (day 93-104)
Participants will be asked to attend the human physiology laboratories after an overnight fast to for a treadmill FAT_{MAX} test (~60 minutes).

Optional visits: a-v difference testing
A subgroup of participants electing to take part in this optional assessment will twice be taken to Birmingham by car for the measurement of arteriovenous (a-v) differences across adipose and muscle. These visits will be scheduled at a convenient time for participants, each less than 10 days before the pre- and post-intervention main trials. These visits are required to take place in the fasted state, with participants encouraged to eat a large meal late in the evening the preceding night (before 23:00). This visit will take 8 hours with travel time, and a meal after the testing.

Procedures

Incremental maximal cardiorespiratory exercise test (FAT_{MAX})

The FAT_{MAX} test is designed to assess peak capacity for fat oxidation and peak oxygen consumption within a single test. This protocol is adapted from Achten et al. [38] with longer (4-min) stages for individuals with lower cardiorespiratory fitness [39]. Participants will arrive in the laboratory after an 8-10 h fast (water only). They will then begin walking on a motorised treadmill at 2 $km \cdot h^{-1}$ with the gradient set at 1% to replicate the oxygen cost of ambulating outdoors. The treadmill speed will be increased by 1 $km \cdot h^{-1}$ every 4 minutes, for 6 stages. After this point, the treadmill speed will remain constant and the gradient will increase by 1% $\cdot min^{-1}$ until volitional exhaustion.

Expired gas samples are collected in the final minute (3-4 minute) of the first 7 stages and a final expired gas sample (between 30-60 seconds) collected when participants give the identified one-minute signal to volitional exhaustion. This final sample allows estimation of $\dot{V}O_2peak$. If a participant continues past this minute, the above process is repeated until volitional exhaustion is reached. The mouthpiece is removed in between stages and provided thirty seconds before each collection to ensure adequate flushing of dead space (excluding final collection). Heart rate (HR) via telemetry (Polar RS400 Heart Rate Monitor, Kempele, Finland) and ratings of perceived exertion (RPE; on a scale ranging from 6 = no exertion to 20 = maximal exertion [40]) will also be recorded during each expired gas sample. Verbal encouragement is provided throughout the test and participants are allowed ad-libitum water intake and use of fans.

Dietary Assessment

Participants will be given a set of kitchen weighing scales and a food recording diary (with instructions) so that they can weigh their food and fluid intake over 3 days. Vitamin D intake will be assessed using a validated food frequency questionnaire [41].

Sleep

Habitual sleep patterns will be monitored objectively using sleep actigraphy before and after the exercise training intervention using a wrist worn MotionWatch (CamNTech, UK). The watch will be worn on the non-dominant wrist continuously for 24 h/day for seven days until they arrive at the laboratory. The MotionWatch is a waterproof, non-invasive, research grade accelerometer, which measures a variety of sleep outcomes including total sleep time, sleep onset latency, and sleep efficiency. The objective assessment of sleep will be undertaken alongside a sleep diary whereby participants will record sleep onset and morning awakening. Participants will also be asked to complete the Morningness-Eveningness Questionnaire (MEQ) before and after the exercise intervention.

Blood sampling

For isolated (single) samples venous blood will be collected by venepuncture. When repeat sampling is required, a cannula (BD Venflon Pro) will be inserted into an antecubital vein for serial blood sampling.

Saliva sampling

Participants will be asked to rinse their mouth with water and then wait 10 minutes before starting the process of sample collection. The participant will then be requested to swallow in order to empty the mouth. Then, to obtain the sample, the participant will remain seated with the head tilted slightly forward and passively dribbling into a pre-weighed sterile tube for 2 min while keeping orofacial movement to a minimum. If there is an insufficient amount of saliva at the end of 2 min, the process will be repeated until an adequate amount is obtained (e.g. 1 ml). The final duration of collection is recorded to calculate saliva flow rate. Participants will be asked not to brush their teeth for at least 45 minutes before saliva collection.

Incidence of upper-respiratory tract infections

Participants will be asked to complete a validated upper respiratory tract infection questionnaire once a week from visit 2 until either visit 6 (comparator group) or visit 12 (RCT). If the answer to the first question is 'no', there are no other items to complete. The questionnaire will be sent to participants electronically using a software system designed for this purpose (Qualtrics). An example of the questionnaire is shown in Appendix 1. Upper respiratory tract infection will be defined as a i) influenza like illness, as indicated by the presence of cough, fever, chilliness, joint aches and pain or ii) a cold verified with Jackson criteria [42]. Scores (ranging from 0-3) for each of the eight Jackson symptoms (sneezing, headache, chilliness, nasal discharge, nasal obstruction, malaise, sore throat, cough) will be summed for each day to calculate a Jackson score. A cold will be defined as total Jackson symptom score of ≥14 and subjective impression of having a cold; total Jackson symptom score of ≥14 and increased nasal discharge for ≥3 days; or total Jackson symptom score of < 14, subjective impression of having a cold and increased nasal discharge score above median run-in nasal discharge score for ≥3 days.

Faecal and urine sampling

Participants will be issued a faeces collection kit and instructions will be provided alongside the participant information sheet. Participants will be asked to provide a faecal sample, collected at their home no earlier than the evening before the trial. Samples collected more than 4 hours before arriving at the University will be refrigerated by the participants. Participants will be asked to provide a urine sample whilst at the University.

Adipose biopsies

Subcutaneous abdominal adipose tissue will be biopsied 4–7 cm lateral of the umbilicus with a 14-gauge needle using the needle aspiration technique [1, 2]. The area will be thoroughly disinfected, before injecting some anaesthetic (approx. 3mL Lidocaine hydrochloride 1%) into a small area. Five minutes later, a needle will be inserted in the subcutaneous fat tissue around the waist in order to collect ~1g of fat tissue.

Muscle biopsies

A muscle sample will be collected from the *vastus lateralis*, using a well-established 'percutaneous muscle biopsy' technique [43]. The area will be thoroughly disinfected with Videne, before injection of some anaesthetic (approx. 5 mL Lidocaine hydrochloride 1%) into a small area under the skin and onto the fascia. Five minutes later, a scalpel will be used to reach and open the fascia. The biopsy needle will then be inserted vertically and the sample will be taken using aspiration. Videne will be used to flood the area and gauze and pressure will be applied to the area immediately once the sample has been collected and maintained for at least 5 minutes. Steri-strips and dressings will then be applied on the area.

Mixed Meal Tolerance Test (MMTT)

The mixed-meal tolerance test (MMTT) will provide ~2636 kJ (~630 kcal) energy, 100 g carbohydrate, 25 g protein and 14 g fat. These amounts will be fixed since the aim is to understand metabolic regulation (e.g., how the metabolic response to an identical challenge has been changed by the intervention). The macronutrient content was chosen on the basis of 100 g carbohydrate should provide a sufficient challenge to blood glucose control for measures of glucose tolerance and insulin sensitivity, whereas the addition of 25 g protein and 14 g fat can both independently alter the physiological response to the meal (thus, their omission would decrease the ability to translate findings to habitual lifestyles [44, 45]).

To further increase the external validity, the MMTT will comprise of 90 g cereal (Crunchy Nut Corn Flakes, Kelloggs, UK) with 550 mL of semi-skimmed milk (Tesco, UK), thus providing a semi-solid meal rather than entirely liquid. The milk will be consumed by pouring 375 mL onto the cereal, and the remaining 175 mL will be consumed as a drink alongside the cereal. Participants will be asked to consume the meal within a 15-minute time frame. Small (6 ml) cannula blood samples will be collected at 15, 30, 45 minutes, 60 minutes and then every 30 minutes for 4 hours.

Stable isotope (tracer) administration

Vitamin D half-life will be measured by giving the participant a low oral dose of a stable isotope of 25OHD: the 'tracer'. We will use stable isotope 25OHD labelled in non-metabolically active positions [46, 47]. Stable isotope compounds occur naturally in the environment and the isotope of vitamin D used for the tracer is naturally present in minute quantities in healthy people. Stable isotopes are nonradioactive, carry no risk for human health, and have been used in a range of groups including pregnant women and children. The higher molecular weight of the stable isotope labelled compound, compared to the more abundantly occurring compound, allows us to measure the labelled compound in human tissues and to distinguish it from molecules already present in the body. The size of the tracer dose is optimised to be just above the lower limit of detection of our analytical method so as to minimally perturb 25OHD status. The dose is 60nmol or 24µg, which is equivalent to the dose in many daily vitamin D supplements. The tracer will be dissolved in 1000 µL olive oil and will be placed on a small piece of bread and given with a standard breakfast (such as buttered toast and a full-fat yoghurt) [46, 47]. The breakfast is standardised and includes some fat to ensure reliable absorption of the tracer. Subjects will be asked not to eat or drink anything (except water) for 3 hours following the dose and the breakfast that is

provided. They will also be asked not to lie down and to refrain from any strenuous physical activity (e.g. sport, or strenuous manual labour) for 3 h following the dose.

There is a degree of flexibility around the scheduling of the blood samples relative to the tracer administration, described below. This is incorporated into the study schematic (Figure 3). Ideally, the 1st and 2nd, and 3rd and 4th, tracer blood samples of each tracer testing period should happen as close to each other as possible (consecutive days), but this is not essential and scheduling should be at the participants convenience.

Event	Day	Flexibility
Dose given to participant	Day 1	-
1st tracer sample	Day 6	+1 or +2 (day 7 or 8)
2nd tracer sample	Day 9	±2 (day 7-11)
3rd tracer sample	Day 27	±2 (day 25 – 29)
4th tracer sample	Day 30	±2 (day 28 – 32)

Adipose arteriovenous (a-v) differences (subset - optional)

We will quantify vitamin D metabolites released from adipose tissue *in vivo* under basal and stimulated conditions using the arteriovenous (a-v) differences technique, which allows tissue-specific blood sampling from human metabolic tissues [48]. Establishing a-v differences will provide quantitative estimates of vitamin D fluxes across adipose tissue. These resource-intensive measures will be performed in a subset of participants transported from Bath to Birmingham (estimated as n=10 lean, n=20 overweight/obese before and after the intervention – 10 intervention and 10 control).

Participants who elect to take part in this part of the a-v assessments will be required to sign a separate specific informed consent form, and undertake the following additional screening steps before being taken to Birmingham:

Resting ECG: A standard 12-lead ECG will be recorded by attaching leads to arms, legs and chest, which will be performed by study staff at the University of Bath. Subsequently, ECG recordings will be reviewed and interpreted by a qualified physician.

Allen Test: The modified Allen test will be used to confirm that the radial artery can be cannulated.

Abdominal vein screening: Due to anatomical differences, not all participants will have suitable veins in abdominal adipose for cannulation. This will be checked using ultrasound.

Vitamin D metabolites, non-esterified fatty acids and glycerol will be measured across subcutaneous abdominal adipose tissue in response to adrenaline infusion, the main physiological stimulus of lipolysis *in vivo* [48]. Thus, these measures will determine whether *in vivo* basal and stimulated release of vitamin D metabolites from adipose tissue is improved with exercise; along with differences between overweight/obese and lean. This aspect of the study also presents a unique opportunity to examine the impact of exercise on skeletal muscle uptake of 25OHD *in vivo*. Significant uptake of 25OHD by skeletal muscle has been reported in cell culture models [49] and this could

have ramifications for skeletal muscle function as well as systemic concentrations of 25OHD. Thus, we will also determine a-v differences for vitamin D metabolites across skeletal muscle *in vivo* through collection and measurement of blood samples from a deep antecubital forearm muscle vein.

Insertion of tissue specific catheters: Suitable veins of the abdomen will be visualised by ultrasound or a cold light source and marked on the skin using appropriate single-use skin markers. Using local anaesthetic (1% lidocaine), catheters will be inserted into a superficial vein draining subcutaneous adipose tissue of the abdomen. Using ultrasound, a suitable deep antecubital muscle vein will be identified, and a catheter will be inserted retrogradely after application of local anaesthetic. A third catheter will be inserted into a radial artery, ideally of the non-dominant hand. All catheter insertions will be performed by a medical doctor in the Clinical Research Facility at the University of Birmingham. After insertion, catheters will be secured with standard dressings and connected to 0.9% NaCl infusion bags. In order to keep lines open, infusion of 0.9% NaCl will be given at 30 ml/hr using a standard infusion pump.

Tissue blood flow ultrasound Doppler measurements: Prior or just after each time point for blood sampling, adipose tissue blood flow will be assessed with ultrasound Doppler. For this, a suitable subcutaneous adipose tissue vein on the abdomen and the leg will be identified with ultrasound. The position of the probe will be marked on the participant's skin using a suitable body marker, in order to minimise variation in the position of the probe at each measurement. Blood flow will be measured using the ultrasound machine's Doppler algorithm over a period of 15 to 30 seconds.

Muscle blood flow measurements: Prior or just after each time point for blood sampling, skeletal muscle blood flow will be assessed with venous occlusion plethysmography. An inflatable cuff will be placed to the arm where the muscle vein catheter is located, and another to the ipsilateral wrist. A special strain gauge will be placed around the lower arm between the two cuffs, secured by medical tape, and will be kept in place throughout the study. During each muscle blood flow measurement the arm cuff will be intermittently inflated and deflated above venous, but below arterial pressure (50 mmHg), whilst the wrist cuff is inflated to supra-arterial pressure (around 180-200 mmHg). Changes in the strain length between inflating and deflating the arm cuff are proportional to the forearm muscle blood flow. Measurements will take less than 2 minutes, at the end of which cuffs are deflated completely.

Adrenaline Infusion: A catheter for infusion purposes will be inserted into an antecubital vein. Following basal sampling (see below), an adrenaline infusion (10 $ng.min^{-1}$.kg fat-free mass) will be given for 30 min. During the infusion, the participants' heart rate will be monitored with a continuous electrocardiogram monitor.

Blood sampling: Blood sampling will be performed simultaneously from all three sites, arterial (ART), adipose (ADI), and muscle (MUS) according to the schedule below (8 ml collected from ART, ADI and MUS, therefore, 24 ml at each time point). After drawing, blood samples are taken on ice immediately to the Clinical Research Facility labs for separation of plasma and storage until further analysis. At each time point, a blood gas syringe is filled (1ml) and analysed using a point of care blood gas analyser.

Table 1: Schedule of blood samples for a-v differences

Time (hrs)	Time (min)	Samples
12:30	-30	ART+ADI+MUS +blood gas from each site
13:00	0	ART+ADI+MUS +blood gas from each site
13:00	Start adrenaline infusion (10 ng.min^{-1}.kg fat-free mass)	
13:30	30	ART+ADI+MUS +blood gas from each site
13:30	Stop adrenaline infusion	
14:00	60	ART+ADI+MUS +blood gas from each site

After the infusion period participants will be provided with a meal before being transported back to Bath.

Blood Sampling volume across the whole study

Appendix 2 shows a summary of the blood samples that will be taken throughout the whole study.

Measurements and Analysis

Vitamin D status and excretion: We will analyse serum levels of multiple vitamin D metabolites as we describe previously [50]; including vitamin D3, 25OHD3, 25OHD2, active vitamin D (1,25-dihydroxyvitamin D, 1,25$(OH)_2$D3) and inactive forms such as 24,25-dihydroxyvitamin D (24,25$(OH)_2$D3) and 3epi-25OHD. Furthermore, since 25OHD circulates bound to the globulin vitamin D binding protein (DBP), and this binding appears to influence the tissue bioavailability and function of 25OHD [51], serum samples will be analysed for concentrations of DBP (and albumin as another non-specific binder of 25OHD)) and free 25OHD using a newly-available ELISA assay from Future Diagnostics (Netherlands). Finally, exercise may also influence the catabolism and excretion of vitamin D metabolites and therefore urine will be collected over 24 h to determine the main vitamin D metabolites. Analysis of urinary vitamin D metabolites has recently been established in Professor Hewison's laboratory (unpublished studies). Collectively, these measures will determine vitamin D 'status', availability and urinary excretion before and after the exercise intervention – and in overweight/obese *versus* lean groups.

Vitamin D turnover and metabolism**:** We will determine 25OHD turnover using the stable isotope tracer. After ingestion of the tracer, blood samples will be collected to determine plasma enrichment of d_3-25OHD_3 by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The half-life ($T_{1/2}$) of 25OHD_3 will be determined as we previously published [52]:

$$T_{1/2} = \frac{\ln(2)}{\kappa_B}$$

where κ_B is the slope of plasma tracer disappearance calculated from the line of best fit of the natural log of plasma d_3-25OHD_3 concentrations versus time from days 7/9 to 28/30 during each tracer period. The use of stable isotopes will provide a dynamic assessment of vitamin D metabolism in response to exercise *versus* control groups plus in overweight/obese *versus* lean.

Vitamin D bioavailability**:** We will assess the impact of exercise on vitamin D bioavailability. Our previous studies have shown that the function of monocytes is proportional to the amount of bioavailable 25OHD in serum [53, 54], and that treatment of vitamin D deficiency improves antimicrobial immunity. Thus, we will use serum from each participant to make individual cell culture preparations (10% donor serum with RPMI medium). These media preparations will then be used to culture monocytes from an anonymous donor, with or without activation via the toll-like receptor 2 (TLR2) agonist 19 kDa lipoprotein, to trigger activation of 25OHD to 1,25$(OH)_2$D. In this way, we will establish whether the change in circulating 25OHD with exercise translates into improved antibacterial (hCAP and other antibacterial proteins) and anti-inflammatory (IL-6, TNFα expression) responses *ex vivo* [53, 54]. This model assesses whether the change in vitamin D is physiologically meaningful. It also has direct biological relevance to adipose tissue given the importance of adipose-resident macrophages in tissue function [2].

Adipose vitamin D content and mobilisation**:** One portion of adipose will be analysed for vitamin D metabolites content as described earlier for serum samples. A second portion will be used to determine vitamin D metabolites in the media of adipose explants cultured *ex vivo* for 3h with and without stimulation (physiological adrenaline). Collectively, these measures will establish whether exercise leads to changes in adipose tissue vitamin D content and the ability to mobilise vitamin D metabolites in basal and stimulated conditions; and whether the ability to mobilise vitamin D metabolites differs in overweight/obese *versus* lean.

Muscle vitamin D content**:** One portion of the muscle biopsy will be analysed for vitamin D metabolites content as described earlier for serum samples to determine whether exercise leads to changes in muscle tissue vitamin D content and whether muscle vitamin D metabolite content differs in overweight/obese *versus* lean.

Serum adipokines and virus/antimicrobial status: We will determine serum concentration of various adipokines, such as Interleukin-6 and leptin, using either individual ELISA assays or a multiplex-type system as previously described [2]. We will use commercial ELISAs to determine IgG antibodies specific for Cytomegalovirus and Epstein-Barr virus. These viruses are known to strongly influence the characteristics of immune cells, especially T cells. We will also use a commercial ELISA to determine concentrations of the antimicrobial peptide LL-37.

Plasma metabolomics and lipidomics: Metabolites will be extracted from plasma as previously described [55]. Metabolite analysis will be carried out using Direct Injection Electospay Ionisation Mass-Spectrometry on a Micromass LCT mass-spectrometer (Micromass/Waters Ltd.). Lipid profiling will involve nano-flow direct infusion high resolution mass spectrometry (nano-DI-HRMS), based on a Q-Exactive Orbitrap (Thermo Scientific, UK), using a Triversa Nanomate (Advion, Ithaca, US).

Adipose and skeletal muscle metabolism: Plasma from arterial and venous blood (a-v difference component) will be used to determine the concentration of metabolites, including non-esterified fatty acids (NEFA), triglycerides, glycerol and glucose (using a clinical chemistry analyser). We will also determine acylcarnitines via liquid chromatography-mass spectrometry (LC-MS).

Adipose tissue transcriptome with targeted protein measurements**:** Exercise leads to a marked improvement in adipose tissue function [17]. This includes pathways involved in lipid mobilisation such as increased hormone sensitive lipase [56] and changes around the lipid droplet that could alter the accessibility of lipid and lipophilic molecules such as increased expression of perilipins [57]. Exercise is likely to change the pathways involved in vitamin D metabolism in adipose tissue given the reported effect of weight loss on vitamin D metabolising enzymes in adipose [58]. Since exercise leads to potentially hundreds of relevant changes we will use RNAseq to avoid overlooking important pathways. We will retain a small portion of adipose to determine the expression of key proteins using western blotting (some established *a priori* but others guided by RNAseq). These measures will establish the pathways involved in improved adipose function and changes in adipose vitamin D metabolism with exercise – plus differences in overweight/obese *versus* lean.

Muscle tissue (other)**:** Exercise leads to profound changes in muscle tissue and this could include pathways involved in the uptake and utilisation of vitamin D metabolites. Thus, we will examine changes in the pathways involved in vitamin D metabolism and other exercise-responsive metabolic/inflammatory pathways either using RNAseq or a more targeted approach (i.e., changes in selected gene expression) with subsequent western blotting.

Mixed Meal Tolerance Test (MMTT): Plasma and serum before and after the mixed meal will be analysed for metabolic (e.g., insulin), appetite (e.g., ghrelin) and inflammatory (e.g., interleukin-6) outcomes.

Adipose tissue immune cell phenotyping: Adipose tissue will be digested using collagenase at 37°C for 60 minutes, followed by centrifugation, lysis of erythrocytes, and addition of fluorophore-conjugated antibodies for identification of adipose-resident immune cells. Adipose tissue immune cells phenotype will be analysed using flow cytometry and compared to identical measurements undertaken in whole blood. Adipocytes isolated following digestion will be stored and used to determine the expression of key genes – to be determined following RNAseq analysis of whole adipose tissue.

Saliva analysis: Saliva supernatants will be analysed for secretory IgA and LL-37 concentration using ELISA. Secretion rates for secretory IgA and LL-37 will be calculated as the multiple of the saliva flow rate and analyte concentration.

Faecal analysis: Faeces will be cultured for major gut bacterial groups. Faecal water will be prepared by centrifugation, and analysed using ‘omic’ technologies (eg. mass-spec and NMR) for presence of metabolites. Faecal samples may also be subject to further analyses using ‘omic’ technologies (eg. mass-spec and NMR) for presence of metabolites. Microbial DNA will be extracted from the faeces, and the composition of the flora will be determined by DNA sequencing on a high throughput sequencing

platform. Patterns of flora composition will be investigated for correlations with clinical data, dietary intake, and health indices. DNA will also be subject to shotgun metagenomic sequencing to determine potential functions of the microbiome.

Other: If our overall study hypothesis is correct and there is evidence that adipose releases more vitamin D or vitamin D metabolites with exercise, we will determine whether this is specific for vitamin D or applies to other lipid soluble molecules found in adipose tissue (e.g., vitamin E, lycopene, dichlorodiphenyltrichloroethane).

Randomisation: 10-week RCT (only)

Overweight participants will be randomly allocated to either exercise intervention or control groups by way of minimisation using age, adiposity, skin colour and Physical Activity Level (PAL) as stratification factors.

Intervention*:* 10-week RCT (only)

Exercise: Participants in the exercise group will undertake a closely-monitored 10-week gym-based exercise intervention commencing between October and January. This duration of intervention is long enough to show major improvements in adipose tissue sensitivity to lipolytic mediators [59, 60] but not too long that follow up measures would be confounded by increased UV exposure in springtime. Exercise will be conducted indoors. The intervention will be progressive and start at a moderate relative intensity with both intensity and duration increased over time similar to our previous intervention studies [29]. By the end of the intervention, participants will be exercising 4 times a week at 70% maximum oxygen uptake. This type of exercise intervention successfully improves adipose tissue function [17, 59, 60]. The prescribed exercise sessions will be supervised and non-exercise physical activity (outside the intervention) will be monitored remotely using a wrist-mounted wearable technology system (MotionWatch 8, CamNtech), which also can collect sleep data to be accompanied with a sleep diary.

A potential confounder during exercise interventions is variability in energy balance and weight loss due to varying degrees of dietary compensation [61]. Thus, we will compensate for the increase in energy expenditure with food prescribed to offset the energy expended during exercise to maintain energy balance. We will monitor energy expenditure through the collection and analysis of expired air samples (indirect calorimetry) during exercise and replace the energy that is expended during exercise sessions with foods containing no vitamin D. Participant weight (body mass) will be recorded every two weeks. Participants will be asked to repeat a 3-d weighed food and fluid record at approximately 6-7 weeks.

Control: The control group will be asked to maintain their normal lifestyle for 10 weeks. They will be offered access to the same exercise programme and access to the fitness facility of their choice at the end of their participation as a route to increase recruitment, retention and compliance. Habitual physical activity will be monitored remotely as in the intervention group with continual MotionWatch 8 wear for the duration of the intervention period. Participants will be asked to repeat a 3-d weighed food and fluid record at approximately 4-5 and 9-10 weeks.

Sample Size

Sample size calculations are based on our preliminary data for the effect of exercise on circulating 25OHD (Figure 1A). With 80% power and an alpha level of 0.05 we will require a sample size of 40 to establish the effect of exercise compared to control (Cohen's *d* = 0.95). This will be increased to N=50 (25:25) to allow for drop out of 20% (drop out was ~17% from our previous longer exercise intervention studies [29]). Based on prior observations, this will provide adequate statistical power to determine meaningful differences between intervention and control groups for our secondary outcomes [1] [2, 46, 62-64]. The reduced sample size for a-v differences in based on both logistical/resource implications plus prior research indicating that sample sizes of 6-10 are sufficient to determine physiologically relevant changes in metabolite fluxes, including fatty acids [48].

Storage & management of data

We will partially process blood, muscle, adipose, urine and faecal samples on the day of the collection before they are frozen in preparation for analysis. Throughout this period and during the subsequent analysis, 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.

On some occasions the analyses may be done in collaboration with a third party including commercial companies, which may require samples to be shipped to this organization (e.g., RNAseq). These samples will be coded and identified only using anonymized sample identifier numbers.

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, and ECG results from the optional a-v difference screening can be provided in a clinically meaningful timeframe. As such, should a participant's data for these measures 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.

Expenses and Benefits

Reasonable travel expenses for visits to the University will be reimbursed on production of receipts or via claims for mileage. Participants who complete the 10-week RCT will be offered a voucher with value of £250 (Love2shop gift vouchers). Participants who complete the 10-week RCT and opt in to travel to Birmingham for the arteriovenous difference measures will be offered a voucher with value of £400. Participants in the comparator group (lean) will be offered a voucher with value of £50 upon completion, and £75 for participating in the a-v difference testing. Participants will be provided with an information pack containing their results for relevant outcomes along with reference ranges (e.g., cardiorespiratory fitness, diet, body composition, blood tests). Participants in the control group of the RCT will also receive access to the fitness facility at the University of Bath for 3-months after completion of the study, and support to undertake an exercise programme during this period (if they so wish).

5 DISADVANTAGES/RISKS AND DISCOMFORT

Participants will be asked to give up their time for repeated visits to undertake various tests. Participants in the exercise group will also be required to regularly visit the University of Bath for exercise training. For some people this may be seen as an inconvenience.

The technique used to acquire muscle biopsies has been in common use in exercise studies since the early 1960s and only minor complications are typically observed (e.g. bleeding from the skin wound, bruising and minor soreness over the days afterwards). Participants are provided with an extensive guide to help manage these issues. This guidance sheet also lists the possible complications which are more severe (e.g. intramuscular bleeding, denervation or infection) although these complications are very rare (the minor complication rate is 0.15% [65]) and risks are minimised by following best practice.

The technique used to take adipose biopsies has also been used since the 1960's. The adipose biopsy site may bleed and is expected to bruise, and bruising can last 2-3 weeks. There is a small chance of localised infection but good practice minimises this risk. All adipose and muscle samples will be taken by specially trained staff who have considerable experience of these techniques.

The use of anaesthetic for biopsies may cause possible side effects including allergic reactions, heart arrhythmia and nausea. However, the initial health screen will be individually checked by a doctor, who will sign a Patient Specific Direction (PSD) to prescribe the anaesthetic for the procedure. A patient information leaflet with further information regarding the anaesthetic will also be included in the participant information sheet.

Venous 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.

During a $\dot{V}O_2$ peak test participants are expected to reach their maximal ability to take up and use oxygen, which by its nature means that they will be exerting a maximal effort. However, participants decide when they can no longer maintain the exercise intensities and, therefore, when the test ends. Participants will be asked to complete the Physical Activity Readiness Questionnaire (PAR-Q) prior to participation in this study. For most people physical activity should not pose any problem or hazard, the PAR-Q has been designed to identify the small number of adults for whom physical activity may be inappropriate. This screen for risk factors should reduce the chance of adverse events (e.g. MI, musculoskeletal injury, etc). Furthermore, the test will be fully supervised by first aid trained staff and the laboratory is equipped with an automated defibrillator for use in the very unlikely event that resuscitation is required.

We will measure body fat, muscle, and bone, using a very sophisticated and precise techniques (dual-energy x-ray absorptiometry (DEXA), and peripheral quantitative computed tomography (pQCT)). DEXA and pQCT are non-invasive radiologic projection techniques that use a very low radiation that is often compared to the exposure to background radiation experienced on daily basis in the UK. For the sake of the present study, with four DEXA scans (including one during screening) and three pQCT scans, this equates to four days 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.

<u>Optional a-v study component (only):</u>

Adrenaline is a drug normally used in emergency situations in hospital. The adrenaline infusion we use (isoprenaline) is very similar to the hormone produced in our body. The infusion is given at a very low dose used widely in research studies, just enough to activate adipose function. During the infusion participants may feel the systemic effects of the infusion: increased heart beat (‘tachycardia’) and a slight discomfort because of blood pressure changes. These effects do not pose any risk and we would minimize them by applying only very small doses of adrenaline. Theoretically, there is a chance that participants could have a serious reaction to the adrenaline. We have been involved in many studies that have used these doses of adrenaline and there have been no such events. However, we will continuously monitor blood pressure and heart rate to ensure participant safety and stop the infusion if there were any signs of discomfort. Any discomfort would cease immediately after discontinuation of the infusion since adrenaline is degraded by our body almost instantly.

The insertion of catheters into the radial artery is widely used in hospital medicine and is considered safe. It is associated with a risk of infection (0.13%), damage to the artery wall (0.09%), local blood collection (14.4%) and bleeding (0.5%). The risk of serious damage to the blood supply of the hand (i.e. artery occlusion) is very low (0.09%). The arterial catheter will be inserted after local anaesthesia and under sterile conditions using ultrasound guidance to minimize the risk of infection and injury. Because of the higher blood pressure in the arteries, the risk of bleeding and bruising is higher compared to the vein catheters. This risk will be much reduced by the use of good clinical practice, and by applying pressure for 15-20 minutes after removal of the catheter.

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

1. Walhin, J.P., et al., *Exercise counteracts the effects of short-term overfeeding and reduced physical activity independent of energy imbalance in healthy young men.* J Physiol, 2013. **591**(24): p. 6231-43.
2. Travers, R.L., et al., *The impact of adiposity on adipose tissue-resident lymphocyte activation in humans.* Int J Obes (Lond), 2015. **39**(5): p. 762-9.
3. Ginde, A.A., et al., *Prospective study of serum 25-hydroxyvitamin D level, cardiovascular disease mortality, and all-cause mortality in older U.S. adults.* J Am Geriatr Soc, 2009. **57**(9): p. 1595-603.
4. Semba, R.D., et al., *Relationship of 25-hydroxyvitamin D with all-cause and cardiovascular disease mortality in older community-dwelling adults.* Eur J Clin Nutr, 2010. **64**(2): p. 203-9.
5. Dobnig, H., et al., *Independent association of low serum 25-hydroxyvitamin d and 1,25-dihydroxyvitamin d levels with all-cause and cardiovascular mortality.* Arch Intern Med, 2008. **168**(12): p. 1340-9.
6. Ruston, D., et al., *The national diet & nutrition survey: Adults aged 19 to 64 years. Nutritional status (anthropometry and blood analytes), blood pressure and physical activity*. 2004.
7. Cashman, K.D., et al., *Vitamin D deficiency in Europe: pandemic?* Am J Clin Nutr, 2016. **103**(4): p. 1033-44.
8. Palacios, C. and L. Gonzalez, *Is vitamin D deficiency a major global public health problem?* Journal of Steroid Biochemistry and Molecular Biology, 2014. **144**: p. 138-145.
9. Vimaleswaran, K.S., et al., *Causal Relationship between Obesity and Vitamin D Status: Bi-Directional Mendelian Randomization Analysis of Multiple Cohorts.* Plos Medicine, 2013. **10**(2).
10. Mawer, E.B., et al., *The distribution and storage of vitamin D and its metabolites in human tissues.* Clinical Science, 1972. **43**(3): p. 413-17.
11. Wortsman, J., et al., *Decreased bioavailability of vitamin D in obesity.* American Journal of Clinical Nutrition, 2000. **72**(3): p. 690-693.
12. Drincic, A.T., et al., *Volumetric Dilution, Rather Than Sequestration Best Explains the Low Vitamin D Status of Obesity.* Obesity, 2012. **20**(7): p. 1444-1448.
13. Thiam, A.R., R.V. Farese Jr, and T.C. Walther, *The biophysics and cell biology of lipid droplets.* Nat Rev Mol Cell Biol, 2013. **14**(12): p. 775-786.

14. Heaney, R.P., et al., *Vitamin D3 distribution and status in the body.* J Am Coll Nutr, 2009. **28**(3): p. 252-6.
15. Didriksen, A., et al., *Vitamin D3 increases in abdominal subcutaneous fat tissue after supplementation with vitamin D3.* European Journal of Endocrinology, 2015. **172**(3): p. 235-241.
16. Di Nisio, A., et al., *Impaired release of vitamin D in dysfunctional adipose tissue: new cues on vitamin D supplementation in obesity.* J Clin Endocrinol Metab, 2017.
17. Thompson, D., et al., *Physical activity and exercise in the regulation of human adipose tissue physiology.* Physiological Reviews, 2012. **92**: p. 157-191.
18. Jocken, J.W.E., et al., *Effect of beta-adrenergic stimulation on whole-body and abdominal subcutaneous adipose tissue lipolysis in lean and obese men.* Diabetologia, 2008. **51**(2): p. 320-327.
19. SACN, *Vitamin D and Health*. 2016.
20. Reinehr, T., et al., *Vitamin D status and parathyroid hormone in obese children before and after weight loss.* European Journal of Endocrinology, 2007. **157**(2): p. 225-232.
21. Tzotzas, T., et al., *Rising Serum 25-Hydroxy-Vitamin D Levels after Weight Loss in Obese Women Correlate with Improvement in Insulin Resistance.* Journal of Clinical Endocrinology & Metabolism, 2010. **95**(9): p. 4251-4257.
22. Rock, C.L., et al., *Weight Loss Is Associated With Increased Serum 25-Hydroxyvitamin D in Overweight or Obese Women.* Obesity, 2012. **20**(11): p. 2296-2301.
23. Wamberg, L., et al., *Expression of vitamin D-metabolizing enzymes in human adipose tissue-the effect of obesity and diet-induced weight loss.* International Journal of Obesity, 2013. **37**(5): p. 651-657.
24. Gangloff, A., et al., *Effect of adipose tissue volume loss on circulating 25-hydroxyvitamin D levels: results from a 1-year lifestyle intervention in viscerally obese men.* International Journal of Obesity, 2015. **39**(11): p. 1638-1643.
25. Gangloff, A., et al., *Changes in circulating vitamin D levels with loss of adipose tissue.* Curr Opin Clin Nutr Metab Care, 2016.
26. Klein, S., E.F. Coyle, and R.R. Wolfe, *Fat metabolism during low-intensity exercise in endurance-trained and untrained men.* Am J Physiol, 1994. **267**(6 Pt 1): p. E934-40.
27. Wolfe, R.R., et al., *Role of triglyceride-fatty acid cycle in controlling fat metabolism in humans during and after exercise.* Am J Physiol, 1990. **258**(2 Pt 1): p. E382-9.
28. Chomistek, A.K., et al., *Vigorous physical activity, mediating biomarkers, and risk of myocardial infarction.* Med Sci Sports Exerc, 2011. **43**(10): p. 1884-90.
29. Thompson, D., et al., *Time course of changes in inflammatory markers during a 6-mo exercise intervention in sedentary middle-aged men: a randomized-controlled trial.* J Appl Physiol (1985), 2010. **108**(4): p. 769-79.

30. Spoo, J.W., et al., *Plasma vitamin D metabolites and C-reactive protein in stage-stop racing endurance sled dogs.* J Vet Intern Med, 2015. **29**(2): p. 519-25.
31. Blum, M., G.E. Dallal, and B. Dawson-Hughes, *Body size and serum 25 hydroxy vitamin D response to oral supplements in healthy older adults.* Journal of the American College of Nutrition, 2008. **27**(2): p. 274-279.
32. Arunabh, S., et al., *Body fat content and 25-hydroxyvitamin D levels in healthy women.* Journal of Clinical Endocrinology & Metabolism, 2003. **88**(1): p. 157-161.
33. Snijder, M.B., et al., *Adiposity in relation to vitamin D status and parathyroid hormone levels: A population-based study in older men and women.* Journal of Clinical Endocrinology & Metabolism, 2005. **90**(7): p. 4119-4123.
34. Beydoun, M.A., et al., *Associations among 25-Hydroxyvitamin D, Diet Quality, and Metabolic Disturbance Differ by Adiposity in Adults in the United States.* Journal of Clinical Endocrinology & Metabolism, 2010. **95**(8): p. 3814-3827.
35. Webb, A.R., L. Kline, and M.F. Holick, *Influence of season and latitude on the cutaneous synthesis of vitamin D3: Exposure to winter sunlight in Boston and Edmonton will not promote vitamin D3 synthesis in human skin.* Journal of Clinical Endocrinology & Metabolism, 1988. **67**(2): p. 373-378.
36. Craig, C.L., et al., *International physical activity questionnaire: 12-country reliability and validity.* Medicine and Science in Sports and Exercise, 2003. **35**(8): p. 1381-1395.
37. Thompson, D., et al., *Assessment of low-to-moderate intensity physical activity thermogenesis in young adults using synchronized heart rate and accelerometry with branched-equation modeling.* Journal of Nutrition, 2006. **136**(4): p. 1037-1042.
38. Achten, J. and A.E. Jeukendrup, *Maximal fat oxidation during exercise in trained men.* Int J Sports Med, 2003. **24**(8): p. 603-8.
39. Chrzanowski-Smith, O.J., et al., *Evaluation of a graded exercise test to determine peak fat oxidation in individuals with low cardiorespiratory fitness.* Applied Physiology, Nutrition, and Metabolism, 2018.
40. Borg, G., *Perceived exertion: a note on "history" and methods.* Med. Sci. Sports Exerc., 1973. **5**(2): p. 90-93.
41. Kiely, M., et al., *Development, validation and implementation of a quantitative food frequency questionnaire to assess habitual vitamin D intake.* Journal of Human Nutrition and Dietetics, 2016. **29**(4): p. 495-504.
42. Martineau, A.R., et al., *Vitamin D3 supplementation in patients with chronic obstructive pulmonary disease (ViDiCO): a multicentre, double-blind, randomised controlled trial.* Lancet Respir Med, 2015. **3**(2): p. 120-130.
43. Perrin, L., et al., *Transcriptomic analyses reveal rhythmic and CLOCK-driven pathways in human skeletal muscle.* eLife, 2018. **7**: p. e34114.

44. Dubois, C., et al., *Effects of graded amounts (0–50 g) of dietary fat on postprandial lipemia and lipoproteins in normolipidemic adults.* The American Journal of Clinical Nutrition, 1998. **67**(1): p. 31-38.
45. Gunnerud, U.J., E.M. Östman, and I.M.E. Björck, *Effects of whey proteins on glycaemia and insulinaemia to an oral glucose load in healthy adults; a dose–response study.* European Journal Of Clinical Nutrition, 2013. **67**: p. 749.
46. Jones, K.S., et al., *Vitamin D expenditure is not altered in pregnancy and lactation despite changes in vitamin D metabolite concentrations.* Scientific Reports, 2016. **6**: p. 26795.
47. Walsh, J.S., et al., *Free 25-hydroxyvitamin D is low in obesity, but there are no adverse associations with bone health.* Am J Clin Nutr, 2016. **103**(6): p. 1465-71.
48. Manolopoulos, K.N., F. Karpe, and K.N. Frayn, *Marked resistance of femoral adipose tissue blood flow and lipolysis to adrenaline in vivo.* Diabetologia, 2012. **55**(11): p. 3029-37.
49. Abboud, M., et al., *Evidence for a specific uptake and retention mechanism for 25-hydroxyvitamin D (25OHD) in skeletal muscle cells.* Endocrinology, 2013. **154**(9): p. 3022-30.
50. Jenkinson, C., et al., *High throughput LC–MS/MS method for the simultaneous analysis of multiple vitamin D analytes in serum.* Journal of Chromatography B, 2016. **1014**: p. 56-63.
51. Chun, R.F., et al., *Vitamin D and DBP: the free hormone hypothesis revisited.* J Steroid Biochem Mol Biol, 2014. **144 Pt A**: p. 132-7.
52. Jones, K.S., et al., *Plasma appearance and disappearance of an oral dose of 25-hydroxyvitamin D2 in healthy adults.* Br J Nutr, 2012. **107**(8): p. 1128-37.
53. Adams, J.S., et al., *Vitamin d-directed rheostatic regulation of monocyte antibacterial responses.* J Immunol, 2009. **182**(7): p. 4289-95.
54. Liu, P.T., et al., *Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response.* Science, 2006. **311**(5768): p. 1770-3.
55. Jones, A.W., et al., *Effects of bovine colostrum supplementation on upper respiratory illness in active males.* Brain, Behavior, and Immunity, 2014. **39**: p. 194-203.
56. You, T., et al., *Effect of exercise training intensity on adipose tissue hormone sensitive lipase gene expression in obese women under weight loss.* Journal of Sport and Health Science, 2012. **1**(3): p. 184-190.
57. Covington, J.D., et al., *Potential Effects of Aerobic Exercise on the Expression of Perilipin 3 in the Adipose Tissue of Women with Polycystic Ovary Syndrome: A Pilot Study.* European journal of endocrinology / European Federation of Endocrine Societies, 2015. **172**(1): p. 47-58.

58. Wamberg, L., et al., *Expression of vitamin D-metabolizing enzymes in human adipose tissue[mdash]the effect of obesity and diet-induced weight loss.* Int J Obes, 2013. **37**(5): p. 651-657.
59. de Glisezinski, I., et al., *Aerobic training improves exercise-induced lipolysis in SCAT and lipid utilization in overweight men.* American Journal of Physiology-Endocrinology and Metabolism, 2003. **285**(5): p. E984-E990.
60. Moro, C., et al., *Training enhances ANP lipid-mobilizing action in adipose tissue of overweight men.* Medicine and Science in Sports and Exercise, 2005. **37**(7): p. 1126-1132.
61. Turner, J.E., et al., *Nonprescribed physical activity energy expenditure is maintained with structured exercise and implicates a compensatory increase in energy intake.* Am J Clin Nutr, 2010. **92**(5): p. 1009-1016.
62. Betts, J., et al., *Bath Breakfast Project (BBP) - Examining the role of extended daily fasting in human energy balance and associated health outcomes: Study protocol for a randomised controlled trial [ISRCTN31521726].* Trials, 2011. **12**(1): p. 172.
63. Betts, J.A., et al., *The causal role of breakfast in energy balance and health: a randomized controlled trial in lean adults.* The American Journal of Clinical Nutrition, 2014. **100**(2): p. 539-547.
64. Chowdhury, E.A., et al., *The causal role of breakfast in energy balance and health: a randomized controlled trial in obese adults.* The American Journal of Clinical Nutrition, 2016. **103**(3): p. 747-756.
65. Tarnopolsky, M.A., et al., *Suction-modified Bergström muscle biopsy technique: Experience with 13,500 procedures.* Muscle Nerve, 2011. **43**: p. 716-725.

7 APPENDICES

APPENDIX 1: Upper respiratory tract infection (URTI) questionnaire

	Day 1 .....
Do you think that are you suffering from the common cold or flu today?	Yes ☐ No ☐
If yes, please indicate which of the symptoms below are present today according to a scale of 0 (no symptoms) to 3 (severe symptoms)	None 0 Mild 1 Moderate 2 Severe 3
Sneezing	0 1 2 3
Headache	0 1 2 3
Feeling generally unwell	0 1 2 3
Runny nose	0 1 2 3
Blocked nose	0 1 2 3
Sore throat	0 1 2 3
Cough	0 1 2 3
Fever	0 1 2 3
Chilliness	0 1 2 3
Joint aches and pains	0 1 2 3

APPENDIX 2: Overview of blood sample volumes

Overview of blood samples and volumes that will be collected throughout the study.

	Visit											SUM
RCT (Overweight)	**2**	**3**	**4**	**5**	**7**	**8**	**9**	**10**	**11**	**a-v 1**	**a-v 2**	
Tracer-related measurements	5	5	5	5	5	5	5	5	5			
A-V										100	100	
MMTT				66					66			
Fasted (vit D)				5					5			
Fasted (vit D bioavailability)				5					5			
Fasted (metabolic & adipokines)				5					5			
Fasted (metabolomics & lipidomics)				1					1			
Fasted (calcium)				1					1			
Fasted (FACs & CMV/EBV)				2					2			
Reserve (back up)				5					5			**426**
Comparator (Lean)	**2**	**3**	**4**	**5**						**a-v 1**		
Tracer	5	5	5	5								
A-V										100		
MMTT				66								
Fasted (vit D)				5								
Fasted (vit D bioavailability)				5								
Fasted (metabolic & adipokines)				5								
Fasted (metabolomics & lipidomics)				1								
Fasted (calcium)				1								
Fasted (FACs & CMV/EBV)				2								
Reserve (back up)				5								**210**

Values are all in ml. The A-V samples are highlighted in red because these are optional (i.e., not all participants will have these measures taken).