Department for Health

UNIVERSITY OF BATH

STUDY PROTOCOL:

Body Composition and Lipid Metabolism at Rest and During Exercise: A Cross-Sectional Analysis

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INVESTIGATORS

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Overview:

Obesity and type 2 diabetes (T2D) represent major global public health challenges. In the United Kingdom alone, more than 55% of the adult population, are predicted to be obese by 2050 [1], while the prevalence of T2D is estimated to increase to 9.5% of the population by 2030 [2]. Moreover, the annual cost of obesity to wider society is estimated to be £27 billion in England, rising to nearly £50 billion by 2050 [3].

Metabolic inflexibility is proposed to be shared characteristic of obesity and insulin resistance [4]. In healthy lean individuals, fat represents the main source of energy during fasting conditions, where in response to feeding switches to predominantly glucose (carbohydrate) oxidation with fat oxidation supressed; this has been coined 'metabolic flexibility' [5]. However, individuals with obesity and T2D reportedly do not display normal 'metabolic flexibility'; despite similar rates of fatty acid uptake, skeletal muscle fat oxidation during post-absorptive resting conditions has been shown to be lower in individuals with obesity and T2D compared to lean individuals [6, 7].

More recently, there has also been a call to further extend the concept of metabolic flexibility to encompass a broader range of paradigms such as substrate use during exercise [8, 9]. Similarly at rest, fat provides an important source of energy during low-to-moderate intensity exercise [10, 11], albeit the contribution of fat is dependent upon numerous factors, including nutritional status [12]. During high-intensity exercise however, carbohydrate is the predominant fuel source, where an ability to switch between oxidising lipids at lower exercise intensities, and carbohydrate at higher exercise intensities, may also reflect an aspect of 'metabolic flexibility'. Nevertheless, fat is clearly an important fuel source during fasting and prolonged exercise. Furthermore, an impaired ability to utilise fat during such conditions is implicated in the pathogenesis of obesity and insulin resistance [5, 7, 8, 13].

Correspondingly, lower fasting rates of fat oxidation have been reported in individuals with obesity compared to lean individuals [7, 14-17]. Furthermore, greater fasting fat oxidation has been associated with lower prospective body weight and fat gain / regain [18-23]. During exercise, high rates of fat oxidation have been associated with reduced post-exercise energy intake / balance [24-26], greater weight loss maintenance [27], and greater exercise-induced fat loss [28]. Importantly, however, this relationship is not consistently observed, with similar [14, 17, 29-40] or higher [17, 37, 41-43] rates of fat use at rest and during exercise reported in individuals with obesity compared to lean counterparts. Moreover, cross-sectional and

prospective associations do not always support the hypothesis that lower fat oxidation is associated with greater body weight / fat mass gain or regain [27, 44-47]. Therefore, despite being commonly believed, it is currently unclear whether lower fat use at rest or during exercise predisposes or is a characteristic of excess adiposity (i.e. obesity).

The inconsistent cross-sectional case-control and prospective findings between body composition and fat oxidation could partly be due to numerous methodological discrepancies between studies such as participant characteristics, matching of comparative groups, the exercise protocol utilised and / or the assessment of body composition, lipid oxidation and cardio-respiratory fitness levels. For example, two recent studies suggest that adiposity may not influence fuel use independently of cardiorespiratory fitness in recreationally active males [34] and females [33], albeit limited by small sample sizes (n = 24 and 14, respectively) and the restricted adiposity range evident (normal and overweight range) [33]. Moreover, adipose tissue distribution, not total levels per se, may independently be associated with substrate metabolism during exercise [32, 48, 49]. Peak fat use during exercise was reportedly lower in females with higher abdominal to lower body fat mass distribution compared to low abdominal: lower body fat mass ratio [48, 49]. However, the females recruited were all categorised as being of 'normal' weight. To our knowledge, no comprehensive data exists on exploring the relationships between body composition (total and distribution of body fat), muscle and adipose tissue function, and whole-body fat oxidation in both post-absorptive resting and exercising conditions, and thus, should be further explored.

In addition to the potential relationship with body composition, fat oxidation at rest and during exercise is known to be influenced by numerous other factors including nutritional status [12, 50, 51], sex [52-54], exercise mode [53], skeletal muscle fibre composition and glycogen content [55] physical activity level (self-reported) [54, 55] and cardiorespiratory fitness [54, 56, 57]. However, there is reportedly large inter-individual variability in fat oxidation at rest [55] and during exercise [54, 55, 58]. While the above factors may partly account for the inter-individual variability reported, large amounts of variance remains apparently unaccounted for e.g. up to 87% in peak fat use during exercise (mg· kg fat free mass^{-1}·min^{-1}) [54]. Furthermore, such previous studies have typically recruited small samples of young, healthy active individuals, who were also predominantly male, limiting the generalisability of previous findings to the wider population. We want to investigate a comprehensive range of novel and unexplored variables (e.g. sex hormone concentrations, and skeletal muscle and adipose tissue characteristics) alongside previously identified 'determinants' of fat oxidation at rest and

during exercise in a wide range of adults. This will provide us with a more holistic and broad view, allowing determinants to be identified independently of a comprehensive range of measured lifestyle, personal, metabolic, genetic and physiological characteristics.

Lastly, this study will also address the intra-individual variability and reliability of fat oxidation at rest and during exercise by asking individuals to complete two identical main trial days 7 – 14 days apart. Intra-individual variability has become a recent topic of great interest in metabolism research [59-61]. The few studies to date that have investigated the intra-individual variability of fat use during exercise are not only confounded by the above recruitment issues, but have also not reported data on an individual level or systematically applied the wide range of reliability statistical methods available [15, 62-68]. Intra-individual variability may partially account for the previously reported inter-individual variability in fat use and thus, only once accounted for can determinants of fat use be more precisely determined. Importantly, this has not previously been considered in determinant studies. Thus, we will apply the entire range of reliability statistical methods available to assess intra-individual variability and when reporting the results of the current study, will plot anonymous individual data alongside group means. This will provide a highly systematic and valid evaluation of the reliability and intra-individual variability of fat use at rest and during exercise alongside drawing firmer conclusions on potential determinants.

Overall, this project will extensively explore fat oxidation in a wide range of 'healthy' and 'at risk of metabolic disease' adults under two proposed metabolic flexibility paradigms; post-absorptive resting and exercising conditions. This will provide important insights into fat use at rest and during exercise, help evaluate current and future research, and aide in the design of future nutrition and exercise interventions to improve human health.

Study Aims / Objectives:

Therefore, the objectives of this study are to comprehensively and systematically explore whether whole-body fat use at rest and during exercise is:

1) Altered in individuals with overweight or obesity compared to lean individuals
2) Associated with a wide range of physiological, metabolic, lifestyle and genetic variables (i.e. potential determinants or health outcomes)
3) The true inter- and intra-individual variation in fat use which will help to more confidently determine the above objectives.
4) Conduct sub-group analyses to explore any moderators on the association between potential determinants and fat use e.g. sex, diet status (e.g. High CHO Vs Low CHO dietary intake), menstrual cycle (females only) and sex hormone thresholds.

Study Design:

This study proposed is an observational, exploratory cross-sectional study that plans to recruit a total of 200 male and female adults aged between 18 – 65 years, who are classed as either 'healthy' or 'at-risk of metabolic disease'. Participants will visit a laboratory at the University of Bath on four occasions. All visits will take place at a Department for Health Laboratory on the University of Bath Main Campus. Please see figures 1 and 2 for a study outline and schematic, respectively.

Following advertisement of the study, interested participants will be asked to contact the Chief Investigator (Mr Oliver Chrzanowski-Smith [CI]) for further information via email/telephone correspondence. If after reading the information sheet, the potential participant is still interested in partaking, they will be invited to attend a preliminary meeting at the University of Bath Main Campus to further discuss the trial (Visit 1).

Visit 1 (approx. 45 minutes)

After the potential prospective participant has read the information sheet, seen the study flowchart / schematic outlining the protocol and study demands, had any questions answered and are able to competently describe back the study to the CI, they will be asked to sign two consent forms (one copy for them, one copy for the CI) if they are still interested in partaking. Study eligibility and health screening (via questionnaires) will then be assessed and if eligible, a short familiarisation process will take place and a demographic and lifestyle questionnaire completed. The familiarisation process involves breathing through a mouthpiece for 5 minutes

and 10 minutes of very low intensity cycling (at 30, 40 and 65-Watts). This is in an attempt to increase the accuracy of data obtained on each main trial day (Visit 2 and 3). Dates will then be scheduled for Visit 2, 3 and 4 and the collection of the preliminary main trial day lifestyle measurements (diet and physical activity monitoring). For female participants who are eumenorrheic or oligomenorrheic and not using contraception (i.e. combined pill, intrauterine device), Visit 2 and 3 will be scheduled to be completed in the same stage of the menstrual cycle via the self-reported menstrual cycle questionnaire.

Visit 2 and 3 (Main Trial Days; approx. 90 minutes)

Preliminary Main Trial Day Lifestyle Measurements:

For the 7-days prior to Visit 2 and at least 2-days prior to Visit 3, the participant will be instructed to:

- Wear a physical activity monitor (Actiheart^{TM})
- Complete a self-weighed diet diary (this includes at least 3 week-days and at least 1 weekend day prior to Visit 2, and the immediate 48-hrs prior to the beginning of each main trial day)
- Record step count via a provided pedometer or personal device (e.g. mobile phone app)

This is to assess participant's physical activity levels, energy expenditure and energy / macronutrient intake. Participants will be asked to replicate their physical activity levels and diet 48-hrs prior to the beginning of each main trial day (Visit 2 and 3). Additionally, they will be asked to avoid any vigorous physical activity for the prior 48 hrs and avoid alcohol consumption for the prior 24 hrs to the beginning of each main trial day.

Main Trial Days:

Visits 2 and 3 are identical. For both of the main trial days, participants will be asked to arrive at the laboratory after fasting for 12 hrs (± 1 hr). The main trial days can take place either in the morning or afternoon, but must be at the same time of the day (± 1 hr) and must take place within 7-28 days of each other. The main trial days will follow the below protocol:

i. Upon arrival to the laboratory, participants will be asked to provide an approximate 10 mL urine sample and void.
ii. Anthropometric measurements (body stature (cm), weight (kg), and waist and hip circumference (cm)) will then be assessed.

iii. After a brief period of rest in a semi-supine position in a resting laboratory, 4 x 5-min resting expired gas samples will then be collected into Douglas Bags to determine resting metabolic rate and substrate use at rest via indirect calorimetry. The International Physical Activity Questionnaire (IPAQ) will also be completed during the initial brief resting period.

iv. A 10 mL blood sample will then be taken via venepuncture for analysis of metabolites and hormones involved in substrate metabolism, and genotyping.

v. After the 10 mL blood sample, the maximal incremental graded cycling test to volitional exhaustion (FAT_{MAX} test) will be completed (see below for the protocol).

Once the FAT_{MAX} test is finished, the main trial day is complete. If this is the first main trial day (Visit 2), participants will then complete the above protocol 7-28 days later. Participants will be asked to maintain their habitual lifestyle between Visit 2 and 3.

Visit 4 (approx. 50 minutes)

Visit 4 will take place between 2 – 7 days after Visit 3. Participants will be asked to arrive to the laboratory after a 12-hr fast at a similar time of the day to visit 2 and 3 (± 1 hr), at least 48-hrs after Visit 3 and having consumed 568 mL of plain water upon waking. Additionally, the participant will be asked to avoid any vigorous physical activity and alcohol consumption for the prior 24 hrs to the beginning of this Visit. After voiding, a dual energy X-ray absorptiometry (DEXA) scan will be performed to assess body composition. After the scan, the **OPTIONAL** skeletal muscle and / or fat biopsy will then be taken (see below for the skeletal muscle and adipose tissue sampling techniques). Once this visit is finished, the participant's involvement in the study is complete. I

Study Participants:

We plan to recruit a total of 200 male and female adults who are 'healthy' or 'at risk of developing metabolic disease'. Participants will be recruited from within the University of Bath and the surrounding areas by email, online and social media platforms (Twitter, University of Bath webpage and callforparticipants.com), poster advertising and word of mouth.

The inclusion criteria is:

- Male or Female
- Aged between 18 – 65 years

- No current or previous history of known cardio-pulmonary, metabolic or musculoskeletal disease
- A body mass index (body mass in kilograms divided by height in metres squared) between 18.9 and 35 kg/m^2.
- All physical activity / fitness levels
- Willingness and sufficiently able to meet the study demands (including the preliminary main trial day measurements and procedures, and maintain their habitual lifestyle (physical activity and diet) across the main study period [Visit 1 to Visit 4]).

Exclusion criteria:

- Taking any medication that may influence study parameters (lipid and / or carbohydrate metabolism)
- Any reported use of substances, other condition, or behaviour deemed either to pose undue personal risk to the participant, or introduce bias into the experiment.
- Any bleeding disorder or taking any medication which impacts blood coagulation
- Any female who is breast-feeding or may be / is pregnant
- Individuals with radio-opaque implants (such as a knee or other joint replacement) or medical devices (such as a pacemaker)

Additional exclusion criteria for individuals who opt in for the optional skeletal muscle and / or adipose tissue biopsy are:

- Known tendency towards keloid scarring
- Known sensitivity or allergy to any local anaesthetic medicines

Study Methodology:

Questionnaires

Participants will complete several questionnaires throughout the study as outlined under 'Study Design' (please see the enclosed documents outlining each questionnaire). These consist of:

a) Health History questionnaire (completed by all participants)
b) Physical activity readiness questionnaire (PAR-Q; completed by all participants)
c) Lidocaine Administration Health Questionnaire (to be completed by participants who opt for the muscle and / or adipose tissue biopsy)

d) Participant Questionnaire (Age, sex, ethnicity, smoking status, dietary patters / special dietary requirements; completed by all participants)
e) Self-reported Menstrual Cycle / Reproductive Status (Females only)
f) Long Form International Physical Activity Questionnaire (IPAQ; [69])

Diet Assessment

In an attempt to standardise upon commencement of trials and analyse potential associations with study parameters, participants' energy and macronutrient intakes will be assessed via a self-weighed diet diary in the 7 days prior to Visit 2 and 3. This must include at least 3 week-days, at least 1 weekend day and the immediate 48-hrs prior to visit 2. Participants will be asked to replicate their diet for 48-hrs prior to each main trial day (i.e. Visit 2 and 3). For Visit 4 participants will be asked to record their evening. Participants will be provided with a set of digital weighing scales (Pro Pocket Scale TOP2KG, Smart Weigh Scales) to accurately weigh food and energy-containing drink products. Diet records will be analysed to estimate energy and macronutrient intakes (Nutritics Ltd., Dublin, Ireland). These diaries will additionally be used to assess study / standardisation compliance.

Physical Activity / Energy Expenditure Assessment

Daily physical activity levels (kcal/day and minutes) will be estimated for the 7 days prior to Visit 2 and 3 through a combined heart rate and accelerometry chest-worn monitor (Actiheart^{TM}; Cambridge Neurotechnology Ltd., Papworth, UK). Individual calibration of the monitor from the recorded HR's in each stage of the respective FAT_{MAX} test was subsequently performed together with inputting the estimated RMR into the Actiheart^{TM} software to increase the accuracy of estimated physical activity energy expenditure [70]. Participants resting metabolic rate (kcal/day) will be estimated through indirect calorimetry from the collection of the 4 x 5-min resting expired gas samples on each main trial day. The thermic effect of food (kcal/day) will be determined by taking 10% of calculated energy intake. Participants physical activity level (PAL) will also be expressed as a ratio by dividing total daily energy expenditure by resting metabolic rate. Additionally, participants will be asked to monitor their step count for the prior 48 hrs to Visit 2 (via a pedometer or personal device) and approximately replicate the number of steps for the 48 hrs prior to Visit 3. The monitoring of steps is to assist in the adherence to the standardisation procedures by providing a 'physical activity marker' for participants. This is in light that Actiheart^{TM} data can only be analysed post-hoc, by which time

if physical activity standardisation was not met, would be too late to re-organise the trial, potentially invalidating results.

Collecting and Analysis of Expired Gas Sample (Indirect Calorimetry)

Expired gas samples will be collected into 100 - 150 L Douglas bags (Cranlea & Hans Rudolph) via a mouthpiece connected to a two-way, T shaped non-rebreathing valve (Model 2700, Hans Rudolph Inc, Kansas City, Missouri). This valve is connected to a 3-way stopcock valve (Hands Rudolph Inc, Kansas City, Missouri) through falconia tubing (Cranlea). Expired gas samples will be analysed for concentrations of oxygen and carbon dioxide through paramagnetic and infrared transduces, respectively (Mini MP 5200, Servomex Group Ltd., Crowborough, East Sussex, UK). The sensors will be calibrated to a two-point low and high calibration (Low: 99.998% Nitrogen, 0% Oxygen and carbon dioxide; High: Balance nitrogen mix, 20.06% oxygen, 8.11% carbon dioxide) using certified, known concentrations (BOC Industrial Gases, Linde AG, Munich, Germany). The sensors will be turned on for 30 minutes prior to each trial. Ambient oxygen and carbon dioxide concentrations will also be noted during collection of expired gas samples to adjust for changes in these concentrations which may have occurred due to global climate change and laboratory contamination [71]. Expired gas samples will be analysed for at least two minutes and until values are stable. Volume and temperature of expired gas samples will be measured using a dry gas meter (Harvard Apparatus) and digital thermometer (HI98509 Checktemp® 1, Hanna Instruments Ltd, Bedfordshire, UK), respectively, during gas evacuation. Ambient temperature, humidity and barometric pressure will be recorded through a weather station (Technoline WS 6730, TechnoTrade Import-Export GmbH, Berlin, Germany).

Substrate use rates will be estimated at rest and during exercise through the stoichiometric equations outlined by Jeukendrup and Wallis [72] including adjustment for the contribution of glycogen metabolism during exercise.

Anthropometric Measurements and Body Composition Analysis (DEXA Scan)

The following anthropometric measurements will be taken: a) Body mass to the nearest 0.1 kg using electronic weighing scales (BC-543 Monitor, Tanita, Tokyo, Japan); b) Body stature to the nearest 0.1 cm using a wall mounted/attached stadiometer (Holtain Ltd, UK) with participants head positioned in the Frankfort plane and after inhalation of a deep breath [73]; c) Body mass index (BMI) calculated as mass in kilograms, divided by the square of participant's stature in metres (kg/m^2); d) Fat mass index calculated as fat mass in kilograms

estimated by DEXA divided by height in metres squared; e) Waist and hip circumference (cm) to the nearest 0.1 cm using a measuring tape (SECA 201, Hamburg, Germany) with participants arms folded across their thorax. Waist circumference is measured at the narrowest point between the 10th Rib and top of the iliac crest at the end of a 'normal' expiration [73]. Hip circumference is measured at the point of greatest posterior protuberance of the buttocks [73]; f) Waist to hip ratio calculated by dividing waist circumference by hip circumference; and g) Body fat % to the nearest 0.1% through the bioelectrical impedance analysis function on the electronic weighing scales (BIA; BC-543 Monitor, Tanita, Tokyo, Japan). Anthropometric measurements will be determined while participants are barefoot and wearing light clothing.

A Dual Energy X-Ray Absorptiometry (DEXA) scan will be performed at Visit 4 (QDR, Discovery W, Hologic, UK). While DEXA was primarily designed to measure bone mineral density, a secondary application offered is a non-invasive, precise estimation of total and localized body composition. Prior to the scan, the device will be calibrated using a quality control material provided by the manufacturer. Participants will be scanned in light clothing (i.e. shorts for men and athletic clothing for women). Participants will be positioned centrally on the scanning bed; with feet spread apart and hands placed in a mid-prone position such that there was a gap between the arms and trunk. This allows regions of interest to be defined by an operator using the software provided by the manufacturer (QDR for Windows, Hologic, UK).

Urine sample analysis

The 10 mL urine sample will be collected into a 50 mL tube (Corning, Corning Inc, USA) and will be analysed for urine specific gravity by a hand-held refractometer (SUR-NE ATEGO, ATEGO Co Ltd, Tokyo, Japan).

Blood sample collection and separation / analysis

Two x 10 mL whole blood samples will be collected from a vein in the antecubital fossa via venepuncture (BD Vacutainer Safety Lok, BD, USA) into a 10 mL syringe (BD Plastipak, BD, Madrid, Spain) by qualified and experienced phlebotomists (primarily Dr Gonzalez & Mr Chrzanowski-Smith). The blood sample on each main trial day will be split equally into a 5 mL ethylenediaminetetraacetic acid-coated tube (K3 EDTA, Sarstedt, Germany) and a 10 mL serum/clotting activator tube (Serum Z/10 ml, Sarstedt, Germany). The EDTA tube will be centrifuged immediately, while the serum tube will be left to stand at room temperature for 20-min before being centrifuged, both for 15 min at 1700 g (3,000 rpm) and at 4°C (Heraeus

Biofuge Primo R, Kendro Laboratory Products Plc., UK). Plasma and serum samples will be extracted from the EDTA-treated and non-treated tubes, respectively, by splitting equally across eight Eppendorf tubes (4 x plasma, 4 x serum) and immediately frozen at – 20°C, before longer-term storage at -80ºC for later batch analysis. These samples will be analysed for the analysis of metabolites and hormones associated with substrate metabolism (e.g. glucose, non-esterified fatty acids, triacylglycerol, adrenaline, estrogen).

The buffy coat layer (consisting of white blood cells) will also be isolated by removing and discarding any remaining plasma in the EDTA-treated tube and then carefully pipetting the buffy coat layer into an Eppendorf. The buffy coat layer will be immediately frozen at -20°C, before longer-term storage at -80ºC for later genotyping analysis of single nucleotide polymorphisms (SNP's). SNP's previously shown to be associated with BMI, waist-circumference and adipose tissue distribution will be analysed for similar relationships and also with substrate metabolism (e.g. FTO [fat mass and obesity associated gene], FFAR1 [long chain fatty acid G-coupled protein receptor]).

Skeletal Muscle Biopsy Procedure (Optional)

The optional muscle sample (approx. 100 – 200 milligrams) will be obtained from the vastus lateralis through the well-established Bergstrom technique at Visit 4. This will be performed by trained and experienced personnel only. The area will be thoroughly disinfected and local anaesthetic (lidocaine) injected using a small 0.4mm wide, 27G needle. After 5 minutes the area will be completely anesthetised and a scalpel will then be used to make a small incision in the skin. A needle will then be used to snip some muscle from 2-5 cm beneath the surface of the skin and a small stitch will be used to close the incision. Muscle samples will be cleaned, processed and frozen immediately for later determination of muscle characteristics related to substrate metabolism e.g. the activity and/or content of proteins (fatty acid transporters, mitochondria, and enzymes), muscle morphology (fibre type composition, intramyocellular lipid content and localisation) and gene expression (mRNA). This will be completed through western blots, immunohistochemical and polymerase chain reaction (PCR) analysis techniques.

Adipose Tissue Biopsy Procedure (Optional)

The optional fat sample (approx. 1-2 grams) will be obtained from the subcutaneous abdominal region (approximately 5 cm from the umbilicus) using a well-established needle

'lipoaspiration' technique at Visit 4. This will be performed by trained and experience personnel only. As with the muscle biopsy, the area will first be thoroughly disinfected before injection of local anaesthetic (lidocaine) to numb the area. A larger needle will then be inserted to collect a small amount of fat tissue (~1g). A syringe will be added to the end of the needle to create a slight vacuum, which will help to collect the sample. There will also be some movement within the anaesthetised area to dislodge fat cells. As taking the fat sample involves using a needle, there is a small risk of infection as with the blood sample via venepuncture, however, this risk if minimised by our strict adherence to best practice. Some people experience a very small amount of bleeding during the hours immediately after the sample has been taken, however you will be closely monitored during the trial day and provided with information regarding best practice to looking after the area. In the days following the sampling, you may notice some bruising and/or a small lump under the skin, however, these will typically return to normal within a few days/weeks, respectively. Adipose tissue will be cleaned, processed and frozen immediately for later determination of gene expression (mRNA) and content and activity of proteins associated with substrate metabolism. The different types of analysis will be performed by PCR, immunohistochemical analysis and western blot techniques.

All biological samples will be stored at -80^{o}C in a freezer(s) located in a locked and secure Department for Health laboratory on the University of Bath Main Campus.

Incremental graded maximal cardiorespiratory cycling exercise test (FAT_{MAX})

The FAT_{MAX} test is an incremental-graded cycling test to volitional exhaustion originally designed upon a previously described and validated protocol ($GE_{35/5}$) in endurance trained cyclists [74]. The FAT_{MAX} protocol that will be utilised in the current study has also been validated in individuals with low cardiorespiratory fitness levels (≥40 mL·min·kg-1 $\dot{V}O_2$peak) [75] and will likely last between 20 – 35 minutes. The FAT_{MAX} tests were adapted in an attempt to more confidently determine peak fat use and the intensity at which this occurs (FAT_{MAX}) in individuals with a wide range of cardiorespiratory fitness levels. All tests will be completed on a mechanically-braked cycle ergometer (Monark Peak Bike Ergomedic 894E, Varberg, Sweden). Figures 3 and 4 depict the two similar FAT_{MAX} protocols that will be used.

The duration of the first seven stages are 4-minutes with approximate 25-watt increments (except for the 10-watt first stage increment in protocol 1; see Figures 3 and 4 for exact watt increments). From stage 8 onwards, stage duration was shortened to 2-minutes together with

greater watt increments between stages (approximate 50-watt increases) until volitional exhaustion was reached. The aim of this adjustment was to twofold; 1) To increase the sensitivity of the protocol to measure / capture peak fat use in the beginning stages and 2) To ensure volitional exhaustion and peak oxygen consumption ($\dot{V}O_2$peak) were quickly attained to reduce the confounding of fatigue. The revolutions per minute (RPM) are 30, 40 and 60 RPM for the first, second and third stage in FAT_{MAX} Protocol 1, and 40 and 60 RPM for the first and second stage in FAT_{MAX} Protocol 2, where a cadence of 80 RPM is then maintained for the remainder of the stages.

To keep the FAT_{MAX} protocols simple and easy to perform for the participant, RPM was standardised to multiples of 10 and kept constant where possible. This lead to slight discrepancies between the intended and actual watts at each stage (see figures 3 and 4). With 0.1kg representing the lowest weight available at the University of Bath facilities, the required applied mass to achieve the desired watts at each stage was not possible when holding RPM constant. Additionally, the weight cage on the Monark Ergomedic 894E peak bike applies a constant mass of 1kg, thus to elicit approximately 30, 40 and 65 watts, the RPM at these stages were lowered. The two protocols were developed upon a validation study of the FAT_{MAX} Protocol 2 [75]. In this study participants who had low cardiorespiratory fitness levels often reached peak fat use in the first stage (40-watts). Thus, a FAT_{MAX} protocol with a first stage of 30-watts was designed in an attempt to more confidently measure peak fat use in individuals with a wide range of cardiorespiratory fitness levels. The FAT_{MAX} test protocol to be completed by the participant will be decided at Visit 1 after their exercise habits have been verbally and written assessed via the health screening and demographic / lifestyle questionnaires. Individuals who self-report in engaging in little or no exercise per week / are physically inactive will complete FAT_{MAX} Protocol 1, whereas regular exercisers will complete FAT_{MAX} Protocol 2. Each individual participant will complete the same FAT_{MAX} Test Protocol on the Main Trial days (Visit 2 and 3) to ensure comparability.

To commence the FAT_{MAX} Test, participants will be set-up on the cycle ergometer through adjustment of saddle and handlebar height and distance to their preferred position. This will then be checked and recorded by the researcher to ensure correct and ensure standardisation for both tests. Participants will then be explained the respective protocol (See figures 3 and 4) and offered the opportunity to ask any questions. A mouthpiece will then be handed to participants for 2-minutes in the position expired gas samples will be collected to accustom

them to breathing through the mouthpiece to reduce the likelihood of hyperventilation and try to ensure data accuracy.

Participants will then be instructed to start cycling at the required RPM (either 30 or 40) and follow the remaining respective protocol until volitional exhaustion. The stop-watch will be started when the RPM of the first stage is reached and stopped immediately upon termination of test. 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 $\dot{V}O_2$peak. If a participant continues past this minute, the above process is repeated until volitional exhaustion is reached. If the final expired gas sample spans across two stages, the intensity will be increased and variance in RPM recorded to appropriately calculate peak power output. The mouthpiece is removed in between stages and provided one-minute before each collection to ensure adequate 'flushing' (excluding final collection). Expired gas samples will be analysed immediately after collection with ambient readings returning to stabilised readings between time-points before the next bag is analysed. Additionally to ensure stabilised ambient gas concentrations are recorded, the Servomex sensor will be left on after analysis of final gas sample until environmental readings are stable with any discordant values (i.e. below these final readings) adjusted for in the indirect calorimetry calculations. 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 [Borg, 1973]) 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. Participants will be asked and reminded throughout the test to keep the cadence constant and as close as possible to the desired RPM. Consistent or repetitive deviations in RPM (+/- 3) will be recorded to appropriately calculate power output.

Sample Size Estimation

A sample size estimation of 200 participants was determined via two methods. Firstly, R2 values (36% and 13%) from two stepwise multiple linear regression models predicting the variance of peak fat use during exercise (g/min and mg/kg Fat Free Mass/min, respectively) in 300 healthy male and female adults were used [54]. Using these R2 values, 42 and 119 participants were required, respectively, to provide a 95% chance of detecting this variation in PFO with α set at 0.05. Secondly, Cohen [76] defines the above effect sizes as large and

medium, respectively. A sample size estimation chart produced by Miles and Shevlin [77] with ten predictors was consulted as a guide. Conservatively, to detect a medium effect size with a power of >95%, approximately 180-190 participants would be required. Taking into account the above, a sample of two-hundred participants was decided upon to account for rolling recruitment, loss of follow-up data due to study withdrawal, non-completers, replacement of drop-outs and any unforeseen issues in data collection.

To minimise the negative impact of loss of follow-up data to validity, participants will be provided with a detailed participant information sheet and have the experimental protocol explained verbally before the onset of the study. They will be advised to carefully consider the required time investment and effort required to complete the study. As such, we hope that exclusions based on likely withdrawals can be accounted for prior to participation. Any unforeseen change in circumstance or withdrawal of consent resulting in loss of follow-up data will be fully documented in the final trial report.

Statistical / Data Analysis

Primary Outcome Measure:	Maximal rate of whole-body fat oxidation (mg/kg FFM/min)
Secondary Outcome Measures:	Maximal rate of whole-body fat oxidation (g/min)
	FAT_{MAX} (% of maximum oxygen consumption)
	FAT_{MAX} (% of Watt max)
	FAT_{MAX} (% of Heart Rate max)
	Resting rate of whole-body fat oxidation (mg/kg FFM/min)
	Resting rate of whole-body fat oxidation (g/min)
	Resting Metabolic Rate (kcal/min)
	$\dot{V}O_2peak$
	Habitual Energy Intake (kcal/day)
	Habitual Macronutrient Intake (grams per day)
	Habitual Physical Activity Energy Expenditure (kcal/day)

	Menstrual Cycle (Self-reported and sex hormone concentrations in blood [Estrogen and Progesterone])
	Fasting measures of blood metabolites: Glucose, Triglycerides, Cholesterol, NEFA, glycerol
	Fasting measures of Adipokines: Leptin, Adiponectin
	Fasting measures of Catecholamines: epinephrine and norepinephrine
	Fasting measures of sex hormones: 17 beta-estradiol, testosterone, progesterone
	Fasting pancreatic derived hormone concentrations: insulin and glucagon
	Hydration Status: Urine Specific Gravity
	Age (years)
	Sex
	Ethnicity
	Smoking Status
	Medication / Supplement Use
	Dietary Patterns / Requirements
	Body Fat Percentage
	Lean Body Mass (kg)
	Body Fat Distribution
	Waist and Hip Circumference
	Fat Mass Index
	Genotype analysis
	Skeletal Muscle Characteristics (Protein Content and / or Activity)
	Adipose Tissue Characteristics (Protein Content and / or Activity)

Data will be checked for normal distribution prior to analysis via Shapiro-Wilk's normality test and visual inspection of histograms. Parametric tests will be conducted on normally distributed data. Non-normally distributed data will be log-transformed if appropriate or the equivalent

non-parametric tests will be conducted. Statistical significance will be set at $P < 0.05$. The statistical analysis for the study objectives identified above are as follows:

1) The effect of body composition on whole-body fat use will be tested by separating groups into defined categories (e.g. lean, overweight or obese) and conducting a one-way ANOVA and mixed design model. The latter to test for any interaction effects across the two main trial days.
2) To identify determinants / characteristics that may be associated with whole-body fat use, correlation analysis will firstly be conducted between whole-body fat use at rest and during exercise with each variable. Statistically significant correlates will then be added to a follow-up hierarchical regression analysis to predict whole-body fat use at rest and during exercise.
3) At a group level, intra-individual variation in whole-body fat use will be tested by a paired sample t-test. A range of further reliability / variability tests will be conducted to comprehensively determine intra-individual variation: Pearson correlation coefficient, Intra Class Correlation, Coefficient of Variation, Bland-Altman plots with 95% Limits of Agreement and Typical Error. Additionally, individual data will be plotted on graphs alongside group means.
4) The inter-individual variation in fat use will be determined by observing the range of whole-body fat use estimates at rest and during exercise recorded after controlling for intra-individual variability / calculated measurement error.

Ethical Considerations / Potential Risks and Discomfort

Skeletal muscle and Adipose Tissue Biopsies: The main ethical issues anticipated relate to the relatively invasive optional skeletal muscle and adipose tissue samples to be acquired at Visit 4. However, as noted, both of these biopsies are optional and thus, participants will be freely able to choose whether or not to have these procedures. The skeletal muscle and adipose tissue biopsies will be obtained to look at associations between fat use at visit 2 and 3, and characteristics of these tissues obtained at Visit 4 (e.g. content and activity of proteins involved in substrate metabolism, skeletal muscle fibre composition and mitochondrial content, location and function). Both techniques are well established procedures in Human Physiology research and are regularly performed in the physiology laboratories at the University of Bath. Only

minor complications are typically observed (such as bleeding from the skin wound, bruising and minor soreness over the days afterwards). For example, the minor complication rate was reportedly 0.15% when using the Bergstrom muscle biopsy technique [78]. Participants will be fully explained the risks associated with the procedures and are provided with an extensive guide (included with the participant information sheet) to help manage these issues if opted for. These sheets also list the possible complications which are more severe (e.g. intramuscular bleeding, denervation or infection) although these complications are fortunately very rare and risks are further minimised by following best practice. All procedures will be performed only by trained individuals to minimize any risk and the minimum amount of each tissue will be taken to minimise discomfort whilst gaining the maximum information to answer the research questions.

Anaesthetic: The use of anaesthetic for muscle and adipose tissue 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.

Dual-energy x-ray absorptiometry (DEXA) Scan: A DEXA is a non-invasive technique that uses a very low exposure to radiation. While DEXA was primarily designed to measure bone mineral density, a secondary application offered is a precise estimate of total and localized body composition. The radiation dose is often compared to the small exposure experienced during a short flight (e.g. London to Paris), and a tiny fraction [1/30th] of the amount of radiation experienced during a typical chest x-ray. This technique is routinely used in hospitals and with elite athletes but nonetheless this does represent some exposure to a small amount of radiation. The risks associated with this amount of radiation are described as 'trivial' (less than 1 in a million per whole body scan). This information will be included in the personalised feedback provided to participants which would not normally be available to them. The exposure of ionising radiation from the DEXA Scan has been reviewed and a declaration made by both a lead Medical Physics Expert (Dr Laura Martin) and a lead Clinical Radiation Expert (Dr Timothy Jenkinson).

Cardiac Event: The incremental graded cycling test to volitional exhaustion (FAT_{MAX} Test), which is performed twice in the study, once at Visit 2 and once at Visit 3, is associated with an

increased risk of a cardiac event. The American College of Sports Medicine [79] report an incident rate of approximately 6 cardiac events per 10,000 tests. This small risk is likely further lowered in individuals with no current or history of cardio-pulmonary or metabolic disease, or family history of cardiac events. This test is central to the aims of the study and all sessions will be closely supervised by first-aid and CPR trained staff. Participants will have the option to freely stop at any stage in the incremental graded cycling tests, further reducing the risk of any cardiac event.

Blood Sampling: Two x approximate 10 mL fasting blood samples across the study will be taken via venepuncture (one at Visit 2 and one at Visit 3). These procedures are associated with a small risk of infection, embolism or haematoma. Further, these procedures can also cause minor bruising and skin irritation. However, the occurrence of such events is very rare and risks are further minimised by our strict adherence to best practice.

Urine Sampling: Two x approximate 10 mL urine samples will be collected across the study (one at Visit 2 and one at Visit 3). This will involve the collection of urine in sealed disposable containers and storage in a fridge until analysis. The samples will be analysed immediately after taken on Visit 2 and Visit 3 with the clinical waste produced disposed as outlined below.

Clinical Waste: As biological samples are being collected, clinical waste will be produced and must be handled appropriately to minimise the risk of injuries and contamination. All contaminated equipment (e.g. needles, syringes, urine sample collection containers) will be immediately disposed of in clinical waste bins or follow strict adherence to 'cleansing protocols' outlined in the Standard Operating Procedures developed by the Department for Health, University of Bath. The clinical waste bins will be sealed (with autoclave) and collected by a member of staff in the Department for Health, University of Bath, before being incinerated in accordance with COSHH policies.

Importantly, risk assessments have been performed and standard operating procedures designed by the Department for Health, University of Bath, are in place for all above procedures and will be strictly adhered too.

Time commitment and inconvenience: Participants will be asked to visit the University of Bath four times (Visit 1 will take approximately 45 minutes, Visit 2 and 3 will take approximately 90 minutes, and Visit 4 will take a maximum of 60 minutes [if both muscle and adipose tissue biopsies are opted for]). Testing will take place after fasting for 12-hrs at a time in the day most suitable for the participant (subject to availability of research team and lab

facilities). Additionally, for lifestyle analysis, participants will be asked to wear a physical activity monitor(s) and fill in a self-weighed diet diary during the 7-days before Visit 2 and Visit 3. Participants will also be asked to replicate their diet and physical activity for at least 48-hrs prior to Visit 2 and Visit 3. For some people this may be seen as an inconvenience, however, participants will be provided with personalised feedback on their habitual activity and diet, which would not normally be available to them.

Consent Issues: Only persons who are capable of understanding the nature of the trial and what it entails will be included in this study (no vulnerable individuals will be included). Even if a person gives written consent to take part in this study they will be made fully aware that they are free to withdraw from the study at any time without giving any explanation. All participants will be fully briefed regarding the nature and risks of all procedures, which are central to the research questions, both verbally and in writing before providing consent to take part in the study.

Data and Sampling Handling / Confidentiality: As personal identifiable data is being collected there is a small risk to participant confidentiality. This will be addressed by generating a unique subject code that will be used on all stored data and samples collected, making it completely anonymous. Furthermore, all hardcopies of identifiable information will be stored in a locked filing cabinet in a secure office on the University of Bath Main Campus. All electronic data / record files will be stored on the University of Bath's secure server, involving a password-protected user log-on and inaccessible to anyone outside of the research team. All biological samples will be stored at -80 degrees C in the Department for Health Physiology laboratory. This laboratory is alarmed and access it limited to the research group. Any published data will be completely anonymised. All personal data and biological samples will be destroyed at the end of the study (defined as 5 years after the completion of the final subject, by which time all analysis must be finished and any remaining samples destroyed). White blood cells from the blood sample (extracted from the buffy coat layer and collected for the isolation of DNA), skeletal muscle samples and adipose tissue samples will be stored under the authority / via approval from the NHS HRA South West – Central Bristol Research Ethics Committee. y held by).

Participant Withdrawal: Participants are freely able to withdraw from the study at any time without providing reasons for doing so and without any prejudice. Participants can withdraw through contacting any member of the research team. If a participant withdraws one month

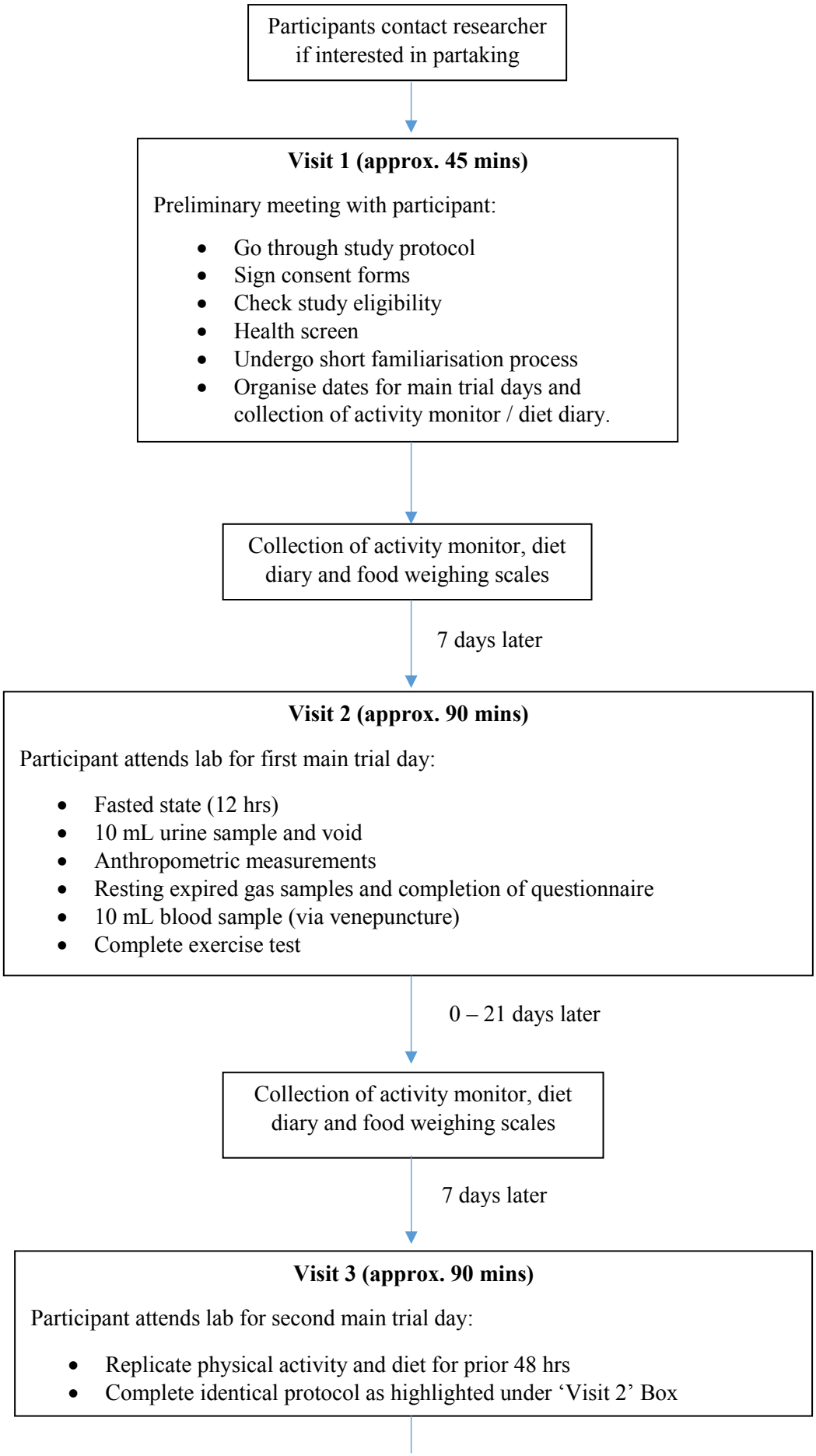
after their completion of the study, it may not be possible to withdraw their individual data as some results may already have been published. This is clearly explained on the participant information sheet and as all data will be anonymised, no individual results will be identifiable in any way.

Advertisement for Study Recruitment: Participants will be recruited through advertising the study using posters, local press (radio and newspaper advertisements), online social media platforms (twitter, facebook, callforparticipants) and the University of Bath website. The poster will be displayed throughout the University of Bath and the local area and with appropriate permission, in other prominent locations (e.g. local sport and fitness centres, community centres, hospitals and GP practices).

Expenses: Participants will receive a free of charge parking permit for the days they are required to visit the University.

Conflicts of Interests**:** There are no conflicts of interest for this study.

Any adverse events relating to the study procedures will be documented and followed up until the event is either resolved or adequately explained.


Participants contact researcher if interested in partaking
Visit 1 (approx. 45 mins)
Preliminary meeting with participant:
Go through study protocol
Sign consent forms
Check study eligibility
Health screen
Undergo short familiarisation process
Organise dates for main trial days and collection of activity monitor / diet diary.
Collection of activity monitor, diet diary and food weighing scales
7 days later
Visit 2 (approx. 90 mins)
Participant attends lab for first main trial day:
Fasted state (12 hrs)
10 mL urine sample and void
Anthropometric measurements
Resting expired gas samples and completion of questionnaire
10 mL blood sample (via venepuncture)
Complete exercise test
0 – 21 days later
Collection of activity monitor, diet diary and food weighing scales
7 days later
Visit 3 (approx. 90 mins)
Participant attends lab for second main trial day:
Replicate physical activity and diet for prior 48 hrs
Complete identical protocol as highlighted under 'Visit 2' Box

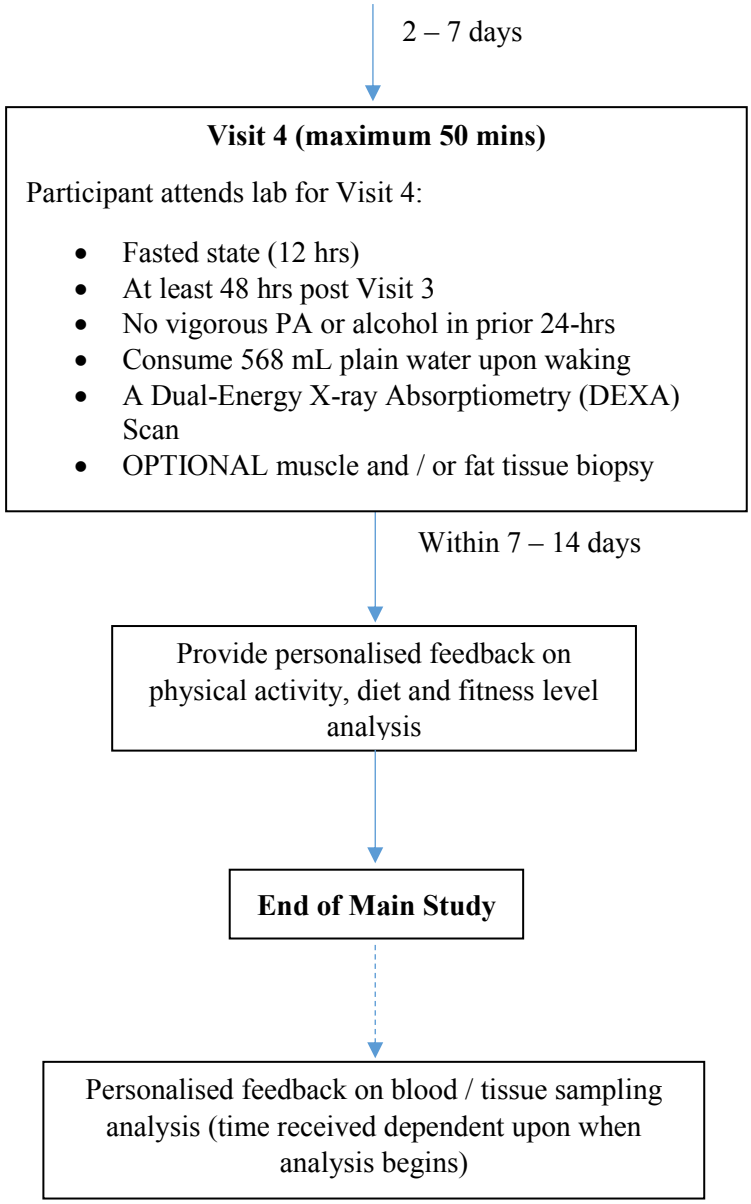
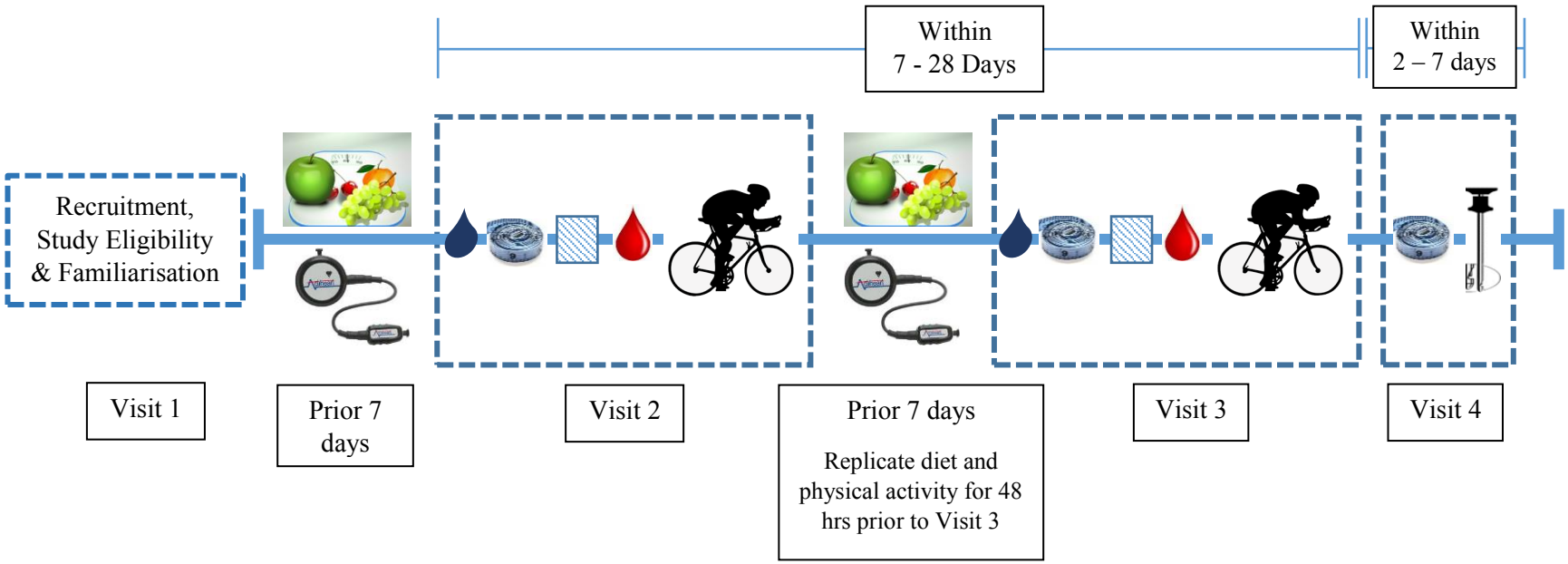


Figure 1. Study Outline and approx. Timelines



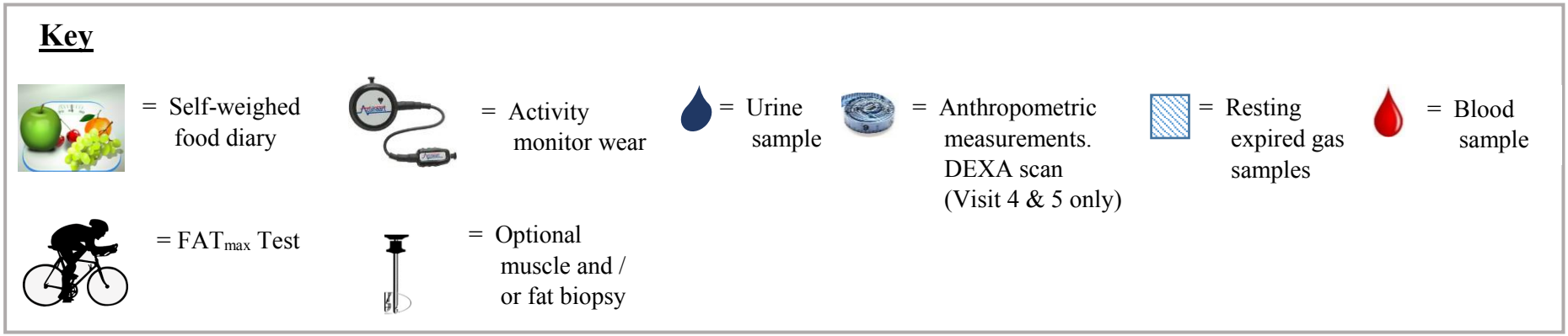


Figure 2. Study Schematic

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	Stage 7	Stage 8	Stage 9	Stage 10	Stage 11	Stage 12	Stage 13	Stage 14
Time (mins)	0 - 4	4 - 8	8 – 12	12 – 16	16 – 20	20 – 24	24 – 28	28 – 30	30 - 32	32 – 34	34 – 36	36 – 38	38 – 40	40 – 42
Applied Mass (kg)	1	1	1.1	1.1	1.5	1.8	2.1	2.7	3.4	4.0	4.6	5.3	5.9	6.6
RPM	30	40	60	80	80	80	80	80	80	80	80	80	80	80
Proposed Watts	30	40	65	90	115	140	165	215	265	315	365	415	465	515
Actual Watts	29.440	39.254	64.769	86.359	117.762	141.314	164.867	211.972	266.927	314.032	361.137	416.092	463.197	518.153
Applied Mass (kg) required for proposed watts	1.019	1.019	1.104	1.146	1.465	1.783	2.102	2.739	3.375	4.012	4.649	5.286	5.923	6.560

Figure 3. FAT$_{MAX}$ Protocol One

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	Stage 7	Stage 8	Stage 9	Stage 10	Stage 11	Stage 12	Stage 13	Stage 14
Time (mins)	0 - 4	4 - 8	8 – 12	12 – 16	16 – 20	20 – 24	24 – 28	28 – 30	30 - 32	32 – 34	34 – 36	36 – 38	38 – 40	40 – 42
Applied Mass (kg)	1	1.1	1.1	1.5	1.8	2.1	2.4	3.1	3.7	4.3	5.0	5.6	6.2	6.9
RPM	40	60	80	80	80	80	80	80	80	80	80	80	80	80
Proposed Watts	40	65	90	115	140	165	190	240	290	340	390	440	490	540
Actual Watts	39.254	64.779	86.359	117.762	141.314	164.867	188.419	243.375	290.480	337.584	392.540	439.645	486.750	541.705
Applied Mass (kg) required for proposed watts	1.019	1.103	1.146	1.464	1.783	2.101	2.420	3.057	3.693	4.330	4.967	5.604	6.241	6.878

Figure 4. FAT$_{MAX}$ Protocol Two

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