The impact of Acipimox and Salbutamol supplementation on the development of insulin resistance and anabolic resistance during forearm immobilization in healthy, young volunteers

Forearm immobilization, metabolic health, and muscle loss

Version 3.4

09-03-2021

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This protocol has regard for the HRA guidance and order of content

RESEARCH REFERENCE NUMBERS

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SIGNATURE PAGE

The undersigned confirm that the following protocol has been agreed and accepted and that the Chief Investigator agrees to conduct the trial in compliance with the approved protocol and will adhere to the principles outlined in the Medicines for Human Use (Clinical Trials) Regulations 2004 (SI 2004/1031), amended regulations (SI 2006/1928) and any subsequent amendments of the clinical trial regulations, GCP guidelines, the Sponsor's (and any other relevant) SOPs, and other regulatory requirements as amended.

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

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

For and on behalf of the Trial Sponsor:

Signature: Date: 09-03-2021

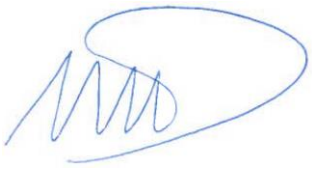
Name (please print): Ms Pam Baxter

Position: Senior Research Governance Officer

University of Exeter

Chief Investigator:

Signature: Date: 09-03-2021



Name:

Dr Marlou Dirks

KEY TRIAL CONTACTS

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i. LIST of CONTENTS

ii. LIST OF ABBREVIATIONS

BCAA	Branched-chain amino acid
BCKDH	Branched-chain alpha-keto acid dehydrogenase
CI	Chief Investigator
CRF	Case Report Form
CSA	Cross-sectional area
CTIMP	Clinical Trial of Investigational Medicinal Product
GCP	Good Clinical Practice
GLP	Good laboratory practice
MHRA	Medicines and Healthcare products Regulatory Agency
PDC	Pyruvate dehydrogenase complex
PI	Principal Investigator
REC	Research Ethics Committee
SAE	Serious Adverse Event
SOP	Standard Operating Procedure
TMF	Trial Master File

iii. TRIAL SUMMARY

Trial Title	The impact of Acipimox and Salbutamol supplementation on the development of insulin resistance and anabolic resistance during forearm immobilization in healthy, young volunteers	
Short title	Forearm immobilization, metabolic health, and muscle loss	
Trial Design	Randomized, double-blind, placebo-controlled trial with three parallel groups	
Study category	Basic science study involving procedures with human participants	
Trial Participants	Young healthy recreationally active males and females	
Planned Sample Size	45	
Treatment duration	2 days of forearm immobilization plus drug or placebo ingestion	
Planned Trial Period	Minimally 12 days (screening, baseline metabolic test day, approximate 7 days waiting period, 2 days forearm immobilization), maximally 33 days (waiting period can be up to 4 weeks, depending on availability of the participant).	
Study sites	Royal Devon & Exeter NHS Foundation Trust Clinical Research Facility (CRF) and Clinical Trials Pharmacy Activities: screening and consenting (CRF), forearm cast application and removal (CRF), metabolic testing (CRF), drug dispensing to study participants (Pharmacy) Nutritional Physiology research laboratories St Luke's Campus, University of Exeter Activities: preparation of a standardised diet, plasma analyses. If required: screening and consenting, forearm cast application	
	Objectives	Outcome Measures
Primary	Determine the impact of reduced muscle lipid availability and increased glycolytic flux on forearm glucose uptake during short-term forearm immobilization in healthy volunteers	Forearm glucose uptake
Secondary	Determine the impact of reduced muscle lipid availability and increased glycolytic flux on muscle protein synthesis, and forearm nutrient balance during short-term forearm immobilization in healthy volunteers	Postabsorptive and postprandial muscle protein synthesis Forearm branched-chain amino acid and fatty acid balance Forearm volume
Medication to be administered	Administered during the 2-day forearm immobilization period: Acipimox OR Salbutamol OR Placebo	
Formulation, Dose, Route of Administration	Acipimox, 250 mg, 4 times daily, oral administration Salbutamol, 4 mg, 4 times daily, oral administration	

	Placebo, no active ingredients, 4 times daily, oral administration
CTIMP? (yes/no)	No (confirmation from MHRA is included in this application)

iv. FUNDING AND SUPPORT IN KIND

FUNDER(S)	FINANCIAL AND NON FINANCIALSUPPORT GIVEN
Wellcome Trust 215 Euston Road London NW1 2BE	Sir Henry Wellcome Postdoctoral Fellowship to Dr Marlou Dirks
Sport and Health Sciences College of Life and Environmental Sciences University of Exeter St Luke's Campus Heavitree Road Exeter, EX1 2LU	

v. ROLE OF TRIAL SPONSOR AND FUNDER

This study is funded by a 4-year Sir Henry Wellcome Postdoctoral Fellowship to Dr Marlou Dirks. Dr Marlou Dirks and Dr Francis Stephens were responsible for the study design. The University of Exeter acts as a sponsor for this study. Dr Marlou Dirks will be responsible for data collection and analysis, decision to publish, and preparation of manuscript(s).

The UK Policy Framework for Health & Social Care and Medicines for Human Use (Clinical Trials) Regulations and Amendment Regulations 2006 require that all health-related research has a sponsor and sets out the responsibilities of the sponsor. The sponsor takes overall responsibility for the initiation, management and financing (or arranging the financing) of the research and must satisfy itself that appropriate arrangements are in place for management, monitoring and reporting of research activity. It is recognised that the sponsor can delegate specific responsibilities to any other individual or organisation that is willing and able to accept them. However, any delegation of responsibilities to another party should be formally agreed and documented by the sponsor.

vii. Protocol contributors

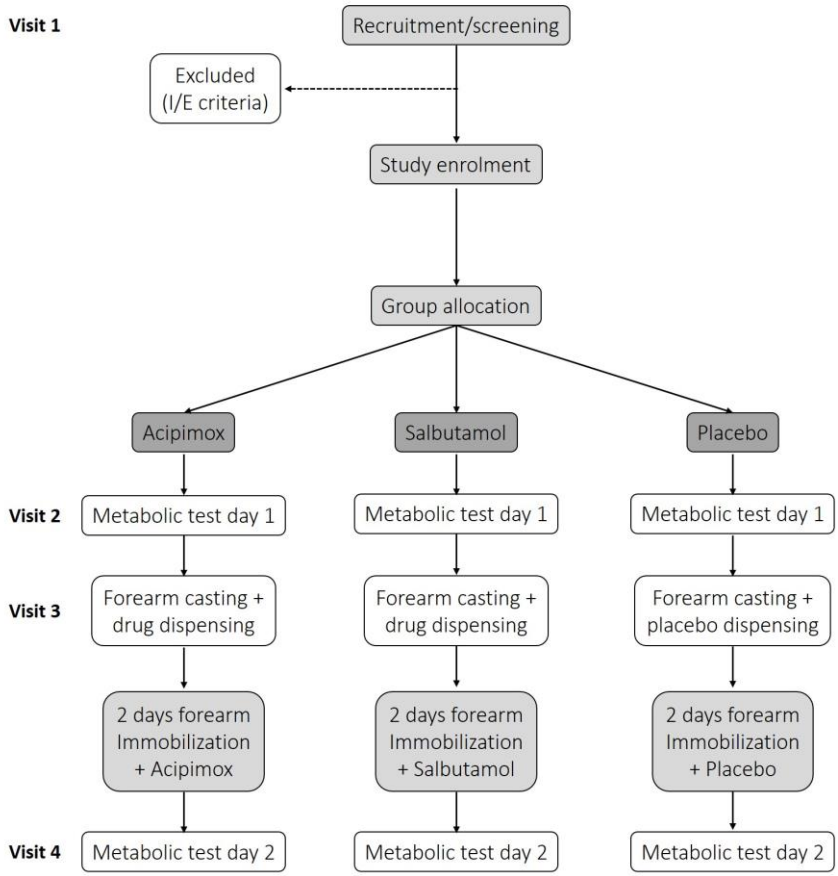
Dr Marlou Dirks – protocol development and scientific critique
Dr Francis Stephens – protocol development and scientific critique
Dr Rob Andrews (medical collaborator) – protocol development and risk assessment
Dr Brad Metcalf – Lead statistician
Dr Jonathan Fulford – MRI Imaging Lead

viii. KEY WORDS:

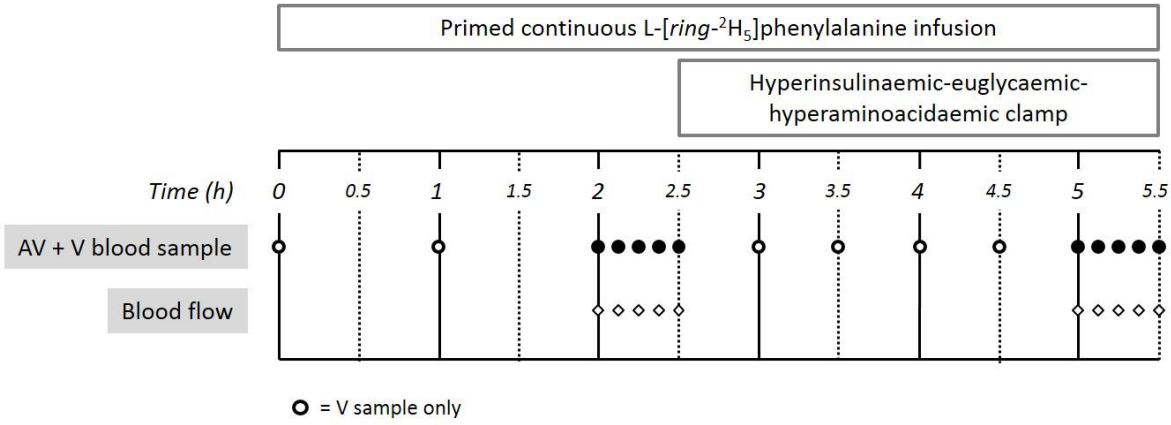
Immobilization, disuse, inactivity, atrophy, insulin resistance, muscle protein synthesis

ix. TRIAL FLOW CHART

Trial flow chart



Schematic overview metabolic test days



1 BACKGROUND

During the course of life, individuals experience multiple bouts of physical inactivity, i.e. muscle disuse. Such periods of for instance illness or injury, which necessitate a period of bed rest or limb immobilisation, lead to a decline in muscle mass of 0.5-0.6% per day [1]. Importantly, older individuals have been shown to have an impaired recovery following such periods of disuse, such that even following a progressive resistance training programme muscle fibre size remains ~11% lower than prior to disuse [2]. As such, the accumulation of such bouts of physical inactivity is thought to contribute substantially to the observed loss of muscle mass with ageing [3, 4]. The occurring muscle atrophy is for a large part due to an impaired muscle protein synthetic response to food ingestion, termed anabolic resistance, a decline which can be as much as 30-55% following short-term immobilisation [5, 6]. In parallel, periods of muscle disuse lead to a deterioration of metabolic health by inducing substantial insulin resistance of glucose metabolism in skeletal muscle tissue. Indeed, following 7 days of muscle disuse, peripheral insulin sensitivity has been shown to decrease by 30-40% [7, 8]. By means of comparison, such a decline in insulin sensitivity is similar to the difference between a normal glucose-tolerant individual and a patient with type 2 diabetes [9], and is equivalent to a decline that is observed following 30–40 years of aging [10, 11]. Importantly, as the decline in muscle mass, strength, and insulin resistance have been shown to be good proxy markers for patient outcomes following hospitalization [12], it is highly important to attenuate or prevent this functional and metabolic decline. To design targeted intervention strategies, insight in the mechanisms underlying muscle disuse atrophy needs to be gained.

Previous work has shown that aberrant muscle fat metabolism is associated with insulin resistance [13]. More specifically, Dr Stephens recently showed that acute lipid infusion, known to induce insulin resistance, simultaneously induces anabolic resistance [14]. Indeed, there are changes in muscle lipid metabolism following 5-7 days of muscle disuse [7, 15], with overt muscle lipid accumulation after 28 days of bed-rest [16] or limb immobilisation [17], suggesting perturbed IMCL turnover may also play a major part of the physiological dysregulation associated with decreased physical activity levels. However, as the development of insulin resistance and anabolic resistance precedes overt skeletal muscle lipid accumulation, it is unclear if perturbed fat metabolism is involved in the development of insulin resistance and anabolic resistance during disuse.

At present, despite decades of physical inactivity research, the causes of disuse-induced insulin and anabolic resistance are still not known. Moreover, we simply do not know whether disturbances in muscle fuel availability and integration can explain the development of insulin resistance and anabolic resistance with muscle disuse. Importantly, if the development of insulin resistance and anabolic resistance can be attenuated or even prevented, this likely has a substantial positive effect on muscle mass retention during disuse. In other words, it will take shorter to get full recovery of muscle mass and muscle strength. Currently, the loss of muscle mass and strength results in protracted loss of function and increased cost on the NHS and Social care. This study will help us to understand how this atrophy occurs. This study will also determine if simple pharmacological interventions are a suitable intervention to prevent muscle loss over the first few days of muscle disuse.

2 RATIONALE

We hypothesize that muscle disuse alters intramuscular glucose and fatty acid metabolism, and their interaction, which consequently leads to decreases in insulin sensitivity and anabolic sensitivity. The primary aim of this study is therefore to determine the impact of reduced muscle lipid availability and increased glycolytic flux on forearm glucose uptake during short-term forearm immobilization in healthy volunteers. A secondary aim is to assess whether these simple pharmacological interventions have an impact on muscle mass loss during the first few days of immobilization; a critical period in a range of clinical scenarios, but one where we still do not know the mechanisms underpinning muscle loss.

We will perform a randomized, double-blind, placebo-controlled study in healthy, young volunteers that will directly characterise the metabolic status of inactive muscle tissue in situations of altered substrate availability. In order to unravel the underlying mechanisms and capture metabolic changes, it is crucial to study the muscle immediately after the onset of insulin resistance. Therefore, participants will undergo 2 days of forearm cast immobilisation, combined with fully-controlled nutritional intake. Prior to and immediately following immobilisation, a metabolic test day will be performed using the AV-V forearm balance technique combined with stable isotope amino acid infusion to quantify muscle glucose uptake (i.e. insulin sensitivity, the primary outcome measure), muscle protein synthesis and breakdown, as well as fatty acid and BCAA balance in the postabsorptive state and during a 3h hyperinsulinaemic-euglycaemic-hyperaminoacidaemic clamp. This clamp will be used as the hyperinsulinaemic-euglycaemic clamp (golden standard for measuring insulin sensitivity) is associated with hypoaminoacidaemia [18], which likely impacts on muscle protein synthesis [19]. By clamping glucose and amino acid concentrations, glucose uptake and muscle protein synthesis can be quantified under steady-state conditions while insulin concentrations are controlled for.

The following interventions will be used to alter muscle substrate availability in a randomised, double-blind, placebo-controlled study with a parallel-group design:

1. Placebo
2. Acipimox, an anti-lipolytic agent which decreases circulating lipids, inhibits intramuscular lipid accumulation and thereby reduces lipid supply to the mitochondria [20].
3. Salbutamol, a selective β2-agonist. Beta2-agonists have been shown to stimulate glycogenolysis and glycolytic flux [21], and exert a hypertrophic effect [22] by increasing muscle protein synthesis and decreasing proteolysis [23, 24].

Our protocol combines the arteriovenous-venous forearm balance technique with stable isotope tracer infusion under hyperinsulinaemic-euglycaemic conditions prior to and following 2 days of forearm immobilization under conditions of altered substrate availability. As such, this study will simultaneously determine the impact of muscle disuse on insulin sensitivity and muscle protein synthesis, and thereby gain crucial insight in the role of these processes, and their interaction, in inactivity-induced muscle loss. It will also determine if simple pharmacological interventions are capable of preventing muscle loss over the first few days of muscle disuse; a critical period in a range of clinical scenarios, but one where we still do not know the mechanisms underpinning muscle loss.

3 ASSESSMENT AND MANAGEMENT OF RISK

A total of 6 venous cannulas will be inserted during the study; 3 on each metabolic test day (one in the elbow for infusion, one in the hand for blood sampling, and one in the elbow for blood sampling). This will involve the insertion of a small needle into the vein to place the cannula. This may cause dizziness and/or nausea if the subject is uncomfortable with needles or blood draws. There is also the possibility of bruising at the needle site. The cannulation will be conducted by trained research nurses and pressure will be applied to the needle site after cannula removal to prevent bruising. Trained first aiders will also be closely available. Per test day, 210 mL blood will be taken over the course of 5.5 hours.

The hyperinsulinaemic-euglycaemic clamp is a procedure routinely used in human physiology research, and Dr Marlou Dirks, Dr Francis Stephens, and Dr Rob Andrews all have significant experience in independently performing this procedure. Co-infusion of an amino acid mixture will be performed to create hyperaminoacidaemic conditions that mimic the postprandial state following food ingestion. This amino acid mixture is a standard product used in clinical care. The main risk of running this procedure is uncontrollable hypoglycaemia. Blood glucose concentrations will be closely monitored every 5 min using a bedside glucose analyser, and the glucose infusion rate will be adjusted accordingly. If blood glucose cannot be maintained, insulin infusion will be stopped immediately, glucose infusion will be further increased, and a carbohydrate-rich drink (present in the room) will be provided to the participant. A minimum of two investigators, of which minimally one of them is emergency first aid-trained, will be in the room at all times. Medical cover will be on call (present in the building) during the insulin infusion.

The acipimox drug may cause some mild-moderate side effects in approximately 2 out-of 10 people. These side effects can include: skin rash; red 'flushed' skin; itching; sensations of hot skin. Less common effects include stomach pain and weakness. The salbutamol drug may cause mild-moderate side effects in approximately 1 out-of 10 people. These side effects van include fine tremor ('shaking', especially of the hands), headache, dizziness, and nausea. Less common side effects can include arrhythmias, low blood pressure, and muscle pain. These reactions usually disappear after 3 days, and failure of symptom reversal in a standard setting normally involves a participant being asked to stop taking the drug. Since participants in the current study are healthy volunteers who will only be taking the drugs for 2 days, no persistent side effects are expected. A clinical doctor (Dr Rob Andrews) will be available for advice throughout the study.

Limb immobilization is a model routinely used to study muscle atrophy in humans, and our previous studies (Dirks et al. 2014 Acta Physiol, Dirks et al. 2016 Acta Physiol, Wall et al. 2013 J Clin Endocrinol Metab, Wall et al. 2014 Acta Physiol, Wall and Dirks et al. 2016 AJP Endo Metab) have used 3-14 days of forearm and leg immobilization in >100 healthy young volunteers without any problems. Forearm immobilization may lead to psychological limitations of having one arm in a cast. During the 2-day immobilization period, participants will likely experience difficulties in performing their daily activities due to only being able to use one arm. A sling will be provided to wear during the day, and a waterproof cover to protect the cast during showering. Any loss or muscle mass and strength that may occur will likely be smaller than 1%, and will restore quickly upon remobilization due to the inclusion of healthy young volunteers.

There will be a time burden on the participant, for which they will receive recompense. The time burden will be spread over a period of around 1.5 weeks, and they will only be required to visit the laboratory for a total of 4 visits. The largest cumulative time burden will be that required to complete the two metabolic test days, approximately 6.5 hours per day.

During the two days of forearm immobilization, participants will be asked to consume a controlled diet and nothing else. Because this diet will be calculated per participant to ensure energy-balanced

conditions, and participants are asked to indicate any food products they cannot or will not eat, the controlled diet should provide adequate dietary intake and not feel as too much of a burden for participants. Of note, this approach has been used in 4 separate studies from the Nutritional Physiology research group, and has proven successful in maintaining energy balance during the studies.

The hyperinsulinaemic-euglycaemic-hyperaminoacidaemic clamp is occasionally associated with gastro-intestinal issues in the last hour of the 3-hour clamp, including vomiting and diarrhoea. To prevent these issues from occurring, we will administer 10 mg metoclopramide hydrochloride intravenously in an already present elbow cannula after 2 hours of the clamp.

Subjects can contact the CI at any time (24/7) if they have concerns or need help. Dr Rob Andrews will be available for advice throughout the supplementation period.

4 OBJECTIVES AND OUTCOME MEASURES/ENDPOINTS

4.1 Primary objective

Determine the impact of reduced muscle lipid availability and increased glycolytic flux on forearm glucose uptake during short-term forearm immobilization in healthy volunteers.

Research question: Does lowering muscle lipid availability and increasing glycolytic flux attenuate the immobilization-induced decline in forearm glucose uptake?
Null hypothesis: Lowering muscle lipid availability and increasing glycolytic flux does not attenuate the immobilization-induced decline in forearm glucose uptake.

3.2 Secondary objectives

Determine the impact of reduced muscle lipid availability and increased glycolytic flux on muscle protein synthesis, and forearm nutrient balance during short-term forearm immobilization in healthy volunteers

3.3 Primary outcome

The primary outcome measure of this study is forearm glucose uptake, as a measure of insulin sensitivity, measured using the arteriovenous-venous forearm balance technique. This will be measured under hyperinsulinaemic-euglycaemic conditions, which is the gold standard for assessing insulin sensitivity *in vivo*.

3.4 Secondary outcomes

Secondary outcome measures include postabsorptive and postprandial muscle protein synthesis, forearm branched-chain amino acid and fatty acid balance, and forearm volume.

3.5 Table of endpoints/outcomes

Objectives	Outcome Measures	Timepoint(s) of evaluation of this outcome measure (if applicable)
Primary Objective Determine the impact of reduced muscle lipid availability and increased glycolytic flux on forearm glucose uptake during short-term forearm immobilization in healthy volunteers.	Forearm glucose uptake	During the steady-state phase of the hyperinsulinaemic-euglycaemic clamp, during the metabolic test days prior to and immediately following 2 days forearm immobilization
Secondary Objectives Determine the impact of reduced muscle lipid availability and increased glycolytic flux on muscle protein synthesis, and forearm nutrient balance during short-term forearm immobilization in healthy volunteers	Postabsorptive and postprandial muscle protein synthesis Forearm branched-chain amino acid and fatty acid balance Forearm volume	During the metabolic test days

5 TRIAL SETTING, DESIGN, AND PROCEDURES

This study is a randomized, double-blind, placebo-controlled trial with a parallel group design in healthy young volunteers. This study will determine the impact of pharmacological manipulation of substrate availability on insulin sensitivity, and anabolic sensitivity during 2 days of forearm immobilization. This study comprises of four study visits, depicted in the study schematic on Page 11 and described in detail in paragraph 5.5 below.

This study is a single-centre study, with all visits performed in the Clinical Research Facility at the Royal Devon & Exeter NHS Foundation Trust. Dispensing of the drugs will be performed by the Clinical Trials Pharmacy at the Royal Devon & Exeter hospital in Exeter.

5.1 Recruitment

Participants will be recruited via convenience sampling. Participants will be recruited via posters, flyers, social media, and word of mouth, from the general public of Exeter and the surrounding areas.

5.2 Screening

Participants will be asked to meet the investigator so that the study can be explained to them in detail, and it is clear what they can expect from the research team and what will be expected of them. Assuming they are happy with what has been discussed, they will then provide written consent. Subsequently, they will complete a medical screening questionnaire and have their height, weight, body composition, and blood pressure measured. All in-and exclusion criteria (please see Section 6) will be assessed. The CI will discuss any issues about eligibility to take part with the medical collaborator.

5.3 Payment

The participant will receive an inconvenience allowance of £125, which will be transferred to their bank account of choice by the University of Exeter finance department. Payment for participants who withdraw from the study or are removed from the study will be dealt with on case-by-case basis.

5.4 Consent

After registering interest, the study will be explained in detail to the participant via email, and they will be given the participant information sheet. They will be asked to carefully read the document, so that they can generate any initial questions. This will occur at least one week in advance of screening. Upon meeting the participant face-to-face the study will be verbally explained in detail. This will include explaining to the participant what they can expect were they to participate, including details of all the procedures involved and time commitments. It will also be made clear what is expected of them. Potential participants will be encouraged to ask any questions they may have. It will be explicitly stated that participants can withdraw at any point during the study without giving a reason, and that they can be afforded time to decide on whether they wish to take part. If they are satisfied, and want to participate in the study, they will subsequently sign the informed consent form (all done under the supervision of an investigator who has undergone informed consent training).
Consent will be sought for surplus muscle samples to be used for ancillary studies subject to additional ethical approval, with the participant having the choice of whether to place restrictions on sample use or not (see informed consent form).

5.5 Trial procedures

Please refer to page 11 for trial flow chart and schematic of the metabolic test days. The trial procedures are described below:

Screening visit (visit 1):

- Firstly, participants will be asked to meet the investigator so that the study can be explained to them in detail, and it is clear what they can expect from the research team and what will be expected of them. Assuming they are happy with what has been discussed, they will then provide written consent. Subsequently, they will complete a medical screening questionnaire and have their height, weight, body composition (Bodpod), and blood pressure measured.
- Participant's caloric requirements will be calculated by estimating Resting Metabolic Rate via the Henry equation, and then multiplying this value by an activity factor derived from the International Physical Activity Questionnaire (IPAQ) questionnaire.
- Forearm volume will be measured via anthropometry.
- Participants will be asked for any allergies or intolerances to ensure they can eat everything they will receive as part of the standardized diet.
- Participants will be provided with a food diary to record their habitual dietary intake for 3 consecutive days, including 2 week days and 1 weekend day.

Baseline metabolic test day (visit 2):

- Approximately 1 week prior to casting, at 8 AM, participants will visit the Clinical Research Facility at the Royal Devon & Exeter NHS Foundation Trust in an overnight fasted state for a metabolic test day.
- The following cannulas will be inserted by a trained research nurse: 1) retrogradely into a deep antecubital vein (draining the muscle bed) of the non-immobilised arm, 2) retrogradely into a superficial dorsal hand vein of the to-be-immobilised hand, and 3) anterogradely into an antecubital vein in the elbow of the to-be-immobilised arm for infusion of insulin, glucose, amino acids, and stable isotope amino acids. The cannulated hand will be placed into a hand-warming unit (air temperature 55°C) to arterialize the venous drainage of the hand.
- After insertion of all cannulas, a primed, continuous stable isotope infusion of L-[*ring*-$^{2}H_5$]-phenylalanine (prime: 0.5 mg·kg^{-1}, infusion 0.5 mg·kg^{-1}·h^{-1}) will be started with participants in a supine position. Using this tracer approach we will be able to calculate muscle protein synthesis and plasma leucine oxidation.
- At t = 2.5 h, a hyperinsulinaemic-euglycaemic-hyperaminoacidaemic clamp will be started. For this, insulin will be infused at a rate of 50 mU·m^{-2}·min^{-1}, and a simultaneous infusion of 20% glucose will be started to maintain euglycaemia at 5.0 mmol·L^{-1}. A 0.3% potassium chloride solution will be infused in the (to be) immobilized arm at a rate of 1 ml/kg body weight/h during the 3-h clamp to maintain plasma potassium concentrations [25]. Hyperaminoacidaemia will be induced by infusion of an amino acid solution (Primene 10%, Baxter) at BCAA concentrations of 700 µmol·L^{-1}. This concentration was chosen as it is the average postprandial BCAA concentration following mixed meal ingestion in the pilot study. Primene will be 7% enriched with L-[*ring*-$^{2}H_5$]-phenylalanine to prevent a decrease in plasma enrichments with Primene infusion. Measurements of blood glucose (Yellow Springs Instruments; every 5 min) and BCAA (spectrophotometric assay, [26]; every 20 min) concentrations will be performed at the bedside, and the infusion rate of glucose and amino acids adjusted accordingly to maintain steady state. Throughout the entire 5.5 h infusion period, repeated arterialised-venous (AV) and deep-venous (V) blood samples will be taken in order to calculate forearm AV-V balance of glucose (i.e. insulin sensitivity), amino acids, and free fatty acids using the Fick principle [27]. Prior to every blood sample, brachial artery blood flow will be measured using Doppler ultrasound.

- After 2 hours of the 3h clamp, a single dose of 10 mg metoclopramide hydrochloride will be administered intravenously in an antecubital elbow cannula, to precent potential gastro-intestinal issues during the final hour of the clamp.
- Following the 3 h clamp, infusion of insulin, amino acids and stable isotope amino acids will be stopped while infusion of glucose will be decreased in a stepwise manner based on blood glucose concentrations. The last 30 min of the clamp will be used to calculate the mean glucose infusion rate (GIR) and mean amino acid infusion rate. Participants will be monitored for minimally 20 min following cessation of the glucose infusion before they are allowed to leave the laboratory.

Forearm casting and drug dispensing (visit 3; to take place between 7 and 28 days after visit 2):

- In the morning at 7:30 AM, participants will visit the Clinical Research Facility at the Royal Devon & Exeter NHS Foundation Trust in an overnight fasted state.
- A fiberglass forearm cast (randomized between dominant and non-dominant arm) will be applied from below the elbow to approximately 2-3 cm below the finger tips, to immobilize the wrist. Participants will be given a sling to wear at all times, except for during sleeping and showering. A waterproof cover will be provided to cover the cast during showering.
- Participants will then receive the food products for the controlled diet, which they will consume during the 2 days of forearm immobilization. Participants will be fed in energy balance, with energy requirements calculated as basal metabolic rate (Henry equations) times an activity factor (IPAQ questionnaire). The diet consists of 1.2 g/kg body weight/day protein (approx. 10-15 en%), 50-55 en% carbohydrate, and 35 en% fat. The diet will be composed of three main meals (i.e. breakfast, lunch, dinner) and snacks, and will contain normal food products. A food diary including recipes for the evening meals will be given, and the entire diet will be explained.
- Lastly, participants will visit the Clinical Trials Pharmacy at RD&E to collect their allocated treatment. Instructions on the times and way to ingest the treatment is provided by trained staff, and a treatment log will be provided. The first out of eight doses will be taken.
- Participants will be sent home, and will return to the CRF two days later for visit 4.

Post-immobilization metabolic test day (visit 4):

- In the morning at 8 AM, participants will visit the Clinical Research Facility at the Royal Devon & Exeter NHS Foundation Trust in an overnight fasted state.
- The metabolic test day will be repeated.
- The fibreglass cast will be removed.

5.6 Randomisation

To determine whether altering muscle substrate availability impacts upon metabolic health, participants will be asked to take an oral supplement 4 times a day during the 2-day forearm immobilization period. Participants will be randomly allocated to receive a supplement containing either acipimox, salbutamol, or a placebo containing only cellulose microcrystalline.

Randomisation will be via a computer generated randomisation schedule provided by the involved statistician (Dr Metcalf), and held in a folder in his office in Richards Building (St Luke's Campus). A secure Excel document of the randomization schedule (treatment A, B, or C) will be accessible by all members of the study team, including Dr Andrews and the Pharmacy contact. Supplementation compliance will be monitored via self-reported supplement intake diaries, and content counting from returned supplement containers.

The arm-to-be-immobilized will be counterbalanced between the dominant and non-dominant arm.

5.7 Blinding

The trial products (i.e. Acipimox, Salbutamol, or placebo) will be received by the Clinical Trials Pharmacy in a distinguishable manner. They will be handled only by unblinded personnel until repackaged in an opaque container. Each container will be labelled with a unique pack ID to identify each pack and its content. The treatment will be prescribed by Dr Rob Andrews, and dispensed to the participant by the Pharmacy. Participants will be instructed not to discuss the product they receive with anyone inside or outside the research team.

5.8 Storage and analysis of clinical samples

Blood samples will be taken throughout the study, as outlined in the study protocol. At each sampling point 8 mL of blood will be taken via cannula, and immediately divided into a plasma (BD Vacutainer - lithium heparin) and serum tube (BD Vacutainer – SST). Plasma samples will be directly snap frozen in liquid nitrogen and analysed for branched-chain amino acids and non-esterified fatty acid concentrations. Serum will be allowed to clot for 30 minutes prior to centrifugation, before being aliquoted into Eppendorf's, snap frozen in liquid nitrogen, and stored in a freezer at -80°C +/- 10°C until analysis for insulin concentration. Plasma L-[*ring*-$^{2}H_{5}$]-phenylalanine enrichments will be measured via gas chromatography-mass spectrometry (GC-MS; [6]). The CI will keep a record of all blood samples taken, of their respective aliquots, and of their location within the freezer.

Samples will be stored for a period of 5 years, in line with HTA guidelines, for further analysis beyond the primary variables depending on the changing landscape of the literature. Samples will be stored in a secure location accessible only via security-card access. Samples will be destroyed either in the process of analysis, or, after the allotted storage period has passed, by being placed in clinical waste and subsequently incinerated. Disposal of plasma and serum will be recorded by the research team in their own archives. Overall responsibility for the samples will reside with Dr Marlou Dirks. Samples will be appropriately labelled in accordance with the trial procedures to comply with the 1998 Data Protection Act. Biological samples collected from participants as part of this trial will be transported, stored, accessed and processed in accordance with national legislation relating to the use and storage of human tissue for research purposes and such activities shall at least meet the requirements as set out in the 2004 Human Tissue Act and the 2006 Human Tissue (Scotland) Act.

6 PARTICIPANT ELIGIBILITY CRITERIA

6.1 Inclusion criteria

- Males and females 18-40 years of age
- Body mass index between 18.5 and 30 kg/m^2

6.2 Exclusion criteria

- Any diagnosed metabolic impairment (e.g. type 1 or 2 diabetes)
- Any diagnosed cardiovascular disease
- Hypertension (≥140 mmHg systolic and/or ≥90 mmHg diastolic)
- Chronic use of any prescribed or over the counter pharmaceuticals (excluding oral contraceptives and contraceptive devices)
- Regular use of nutritional supplements
- A personal or family history of thrombosis, epilepsy, seizures or schizophrenia
- Any previous motor disorders
- Any known disorders in lipid metabolism
- Any known disorders in muscle metabolism
- Known allergy for Acipimox, Salbutamol, or other substances in the tablets
- Known sensitivity for sympathomimetic drugs
- Known hypokalaemia
- Presence of an ulcer in the stomach or gut and/or strong history of indigestion
- Known severe kidney problems
- Previous reaction to metoclopramide
- Known phaeochromocytoma (tumour that secretes noradrenaline or adrenaline) or past history of one
- Recent abdominal surgery
- Pregnancy
- Unable to give consent

7 TRIAL TREATMENTS

The present study is a basic physiology study, which aims to elucidate mechanisms underlying muscle atrophy by studying muscle metabolism in conditions of pharmacologically altered substrate availability. Participants will be randomized to receive one of two drugs (Acipimox and Salbutamol) or a placebo, to be taken orally during the 2-day forearm immobilization period. Products will be prescribed by Dr Rob Andrews, after thorough checking for the listed in- and exclusion criteria (Section 6).

All products will be taken 4 times daily, with or after the three main meals (i.e. breakfast, lunch, dinner), and before bed.

Participants will be able to contact the CI 24/7, and Dr Rob Andrews will be available for advice throughout the supplementation period.

The study products are described below:

Acipimox

The drug Acipimox is a nicotinic acid analogue that inhibits adipose tissue lipolysis, lowers intramuscular lipid metabolites, and improves insulin sensitivity. Acipimox will be given in 250 mg capsules, taken 4 times daily, as this dose has been shown to improve insulin sensitivity in type 2 diabetes patients within a few days [20].
Acipimox may cause some mild-moderate side effects in approximately 2 out-of 10 people. These side effects can include: skin rash; red 'flushed' skin; itching; sensations of hot skin. Less common effects include stomach pain and weakness.

Salbutamol

The drug Salbutamol is a β2-adrenoceptor agonist commonly used as a bronchodilator in asthma. In addition, β2-agonists have been shown to stimulate glycogenolysis and glycolytic flux [21], and exert a hypertrophic effect [22] by increasing muscle protein synthesis and decreasing proteolysis [23, 24]. Salbutamol will be given in 4 mg tablets, taken 4 times daily, as this dose has been shown to lead to a significant increase in lean mass in [22].
Salbutamol may cause mild-moderate side effects in approximately 1 out-of 10 people. These side effects van include fine tremor ('shaking', especially of the hands), headache, dizziness, and nausea. Less common side effects include arrhythmias, low blood pressure, and muscle pain.

Placebo

The placebo supplement will contain lactose, microcrystalline cellulose, pregelatinised starch, sodium starch glycolate, and magnesium stearate. This are inert substances widely used in many pill and tablet formulations, and are unlikely to cause toxicity when taken orally.

8 ADVERSE EVENT RECORDING AND REPORTING

8.1 Definitions

An Adverse Event (AE) is any unfavourable and unintended sign, symptom, syndrome or illness that develops or worsens during the period of observation in the study.

An AE does include a / an:

1. exacerbation of a pre-existing illness.
2. increase in frequency or intensity of a pre-existing episodic event or condition.
3. condition detected or diagnosed after medicinal product administration even though it may have been present prior to the start of the study.
4. continuous persistent disease or symptoms present at baseline that worsen following the start of the study.

An AE does not include a / an:

1. medical or surgical procedure (e.g., surgery, endoscopy, tooth extraction, transfusion); but the condition that leads to the procedure is an AE.
2. pre-existing disease or conditions present or detected at the start of the study that did not worsen.
3. situations where an untoward medical occurrence has not occurred (e.g., hospitalisations for cosmetic elective surgery, social and / or convenience admissions).
4. disease or disorder being studied or sign or symptom associated with the disease or disorder unless more severe than expected for the participant's condition.
5. overdose of concurrent medication without any signs or symptoms.

A Serious Adverse Event (SAE) is any adverse event occurring following study mandated procedures, having received the treatment or intervention that results in any of the following outcomes:

1. Death
2. A life-threatening adverse event
3. Inpatient hospitalisation or prolongation of existing hospitalisation
4. A disability / incapacity
5. A congenital anomaly in the offspring of a participant

Important medical events that may not result in death, be life-threatening, or require hospitalisation may be considered a serious adverse event when, based upon appropriate medical judgment, they may jeopardize the patient or participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition

All adverse events will be assessed for seriousness, expectedness and causality:

A distinction is drawn between serious and severe AEs. Severity is a measure of intensity whereas seriousness is defined using the criteria above. Hence, a severe AE need not necessarily be serious.

8.2 Recording and reporting of adverse events

Any adverse effects noted will be recorded in the adverse event log and reported to the CI and the study sponsors as per standard University of Exeter sponsor protocols (please refer to Letter from sponsor, included in this application). The CI will have a professional obligation to closely monitor participants and in the event of an adverse event being identified, follow established clinical treatment or referral protocols.

Participants will be instructed to contact the study team immediately should they feel unwell, and will be provided with contact numbers for contacting the team directly. Participants will be issued with a

card indicating their participation. This card will also identify their allocation code and identify reasons and who to contact should breaking the allocation code become necessary.

8.3 Emergency unblinding

The investigators may identify the treatment for each participant through sealed envelopes in the event of a medical emergency or SAE when treatment is dependent on knowledge of the actual drug received. The CI will be notified if the treatment code for a participant is broken, and the date and the reason(s) for breaking the blind will be noted in the CRF in a timely manner (notification of Sponsor immediately as practicable by phone or fax, followed by a written narrative of the event within 24 hours, as described above). Emergency unblinding will be carried out by the medical collaborator (Dr Rob Andrews). He will at all times have access to the sealed list with participant names and treatment allocation.

9 STATISTICS AND DATA ANALYSIS

9.1 Sample size calculation

We have powered the study to detect a difference in forearm glucose uptake (primary outcome measure) between 1) Acipimox vs placebo, and 2) Salbutamol vs placebo. We have powered the study based on detecting differences in the change in forearm glucose uptake as measured during the hyperinsulinaemic-euglycaemic clamp, which is golden standard to measure insulin sensitivity. We have recently generated pilot data to demonstrate that forearm glucose uptake decreases by 13±8% (mean±SD) in response to mixed meal ingestion following 2 days of forearm immobilization. Based on our hypotheses, lowering lipid availability (via Acipimox) and stimulating glycolytic flux (via Salbutamol) will alleviate insulin resistance (i.e. prevent the decline in forearm glucose uptake). The following data were used to estimate the effect size, using $\alpha = 0.05$; power = 80%; significance = 5%:

1) Placebo (-13±8%) vs Acipimox (0±8%; preservation of forearm glucose uptake [14]. Effect size: 1.625. Required: *n*=8
2) Placebo (-13±8%) vs Salbutamol (-4.3±6%; 66% attenuation of decline in forearm glucose uptake by a beta2-agonist [28, 29]. Effect size: 1.23. Required: *n*=12

To have enough power for both comparisons, *n*=12 participants will be included in all groups (i.e. placebo, Acipimox, Salbutamol).

Thus, assuming a ≤20% dropout rate (in our experience, similar immobilization studies do not exceed this drop-out rate when using healthy adults), we will recruit 45 healthy adults 18-40 years old, from both sexes, who will be split across 3 parallel groups (i.e. *n*=15 per group, ensuring completion of *n*=12 per group).

Sample size was calculated using G*Power 3.1.9.2.

9.2 Planned recruitment rate

The study will recruit 45 (taking into account a 20% dropout rate, see paragraph 10.1), young healthy males and females, primarily from the University student population. The planned recruitment rate is 2 participants per month over a 24 month period. Participants will be recruited at a single centre, and we foresee no issue capturing the eligibility criteria. We expect a screening failure rate not exceeding 50%, based on previous experience.

9.3 Statistical analysis plan

9.3.1 Summary of baseline data and flow of participants

Key baseline data will include anthropometric data (height, weight, BMI, fat mass), glucose and insulin concentrations, forearm glucose uptake, postabsorptive and postprandial muscle protein synthesis, forearm muscle volume, and forearm branched-chain amino acid (BCAA) and fatty acid balance. All baseline variables will be analysed using One-Way ANOVA to determine whether there are differences between groups.

A CONSORT flow diagram will be produced following completion of the study, and published in the manuscript(s) when possible.

9.3.2 Primary outcome analysis

The primary outcome measure for this study is forearm glucose uptake, measured during the steady-state phase of a 3-h hyperinsulinaemic-euglycaemic-hyperaminoacidaemic clamp. A two-way ANOVA, with and treatment (placebo vs Acipimox vs Salbutamol) as between-subjects factor and time (pre vs post immobilization) as within-subjects factor. A Bonferroni post-hoc test will be used to locate individual differences.

9.3.3 Secondary outcome analysis

Secondary outcome measures include postabsorptive and postprandial muscle protein synthesis, and forearm branched-chain amino acid and fatty acid balance. These data will be analysed in the same way as the primary outcome described above.

9.3.4 Procedure(s) to account for missing or spurious data

The study will be designed to a high level of rigour, and processes will be methodically applied to minimise the occurrence of missing data. Where data are missed due to participant error / non-compliance or technical error, there will be a follow up investigation carried out by the research team try to locate the data, or, failing that, to optimise future data collection. If data are missed due to researcher error or technical failure, then this will be recorded on data sheets available to the research team at all times. Missing data analyses will be carried out using a commercial statistical analysis package (GraphPad Prism 7.03).

10 DATA MANAGEMENT

10.1 Source data and documents

All source data and source documents collected during the trial will be retained to allow subsequent reconstruction and evaluation of the trial. Researchers will ensure that all source data and documents are accurate, legible, complete and consistent to allow verification of the process for the purposes of confirmation, quality control and audit. Personal details will be stored up to a period of 5 years from the completion of the study, as we would like to store the personal details at least in line with the storage of blood samples to allow maximum data generation from the study performed.
Dr Marlou Dirks will act as custodian, and only her and Dr Francis Stephens will have access to the source data. All members of the research team will comply with the Data Protection Act 1998 with regards to the collection, storage, processing and disclosure of personal information and will uphold the Act's principles.
Trial conduct may be subject to systems audit of the Trial Master File (TMF) for inclusion of essential documents; permissions to conduct the trial; Trial Delegation Log; CVs of trial staff and training received; local document control procedures; consent procedures and recruitment logs; adherence to procedures defined in the protocol (e.g. inclusion / exclusion criteria, correct randomisation, timeliness of visits); adverse event recording and reporting; accountability of trial materials and equipment calibration logs.

10.2 Data areas and data types

The research data to be managed are principally quantitative in nature obtained from investigation of healthy young male and female participants (18-40) e.g. screening data (height, weight, BMI, age, blood clinical chemistry, blood pressure, forearm volume etc.), habitual diet, body composition and muscle mass obtained from BodPod, and muscle protein synthesis rates and plasma tracer enrichments and/or amino acid and fatty acid concentrations of the collected blood samples. A proportion of the data will be qualitative (e.g. screening and physical activity questionnaires and diet diaries).

10.3 Standards and metadata

In compliance with UK Data Protection Laws, subject identifiable data (screening data, consent forms and screening questionnaires), and all hard copies of lab notebooks and documents will be kept in a locked room within the Clinical Research Facility (RILD building, RD&E site), specifically dedicated to storage of identifiable data. Once enrolled into a study, participants are assigned a subject number and research data generated incorporate this subject number as an identifier, i.e. no direct link to participant identity is used. Encrypted and password-protected electronic copies of these data will be made to ensure against the risk of loss. All members of the research team have relevant expertise of information security; having received training within the host institution.
All forms of experimental data will be collected electronically onto computer hard drives intrinsic to the equipment being used to make the measurement in question (e.g. mass spectrometer). Sufficient storage space is available on each system to allow adequate storage of large raw data, but all data will be backed up onto the project specific computer. Where possible the project will use open or widely used data formats and files. Electronic data will subsequently be stored on University managed network drives, featuring data backup and redundancy systems, with access rights to the storage area restricted to key personnel. Each researcher is allocated up to 100GB of secure, backed up network storage. Any data stored on the CI's computer will be backed up daily to an encrypted external hard drive, stored in a geographically different location, before being transferred to the network drive at the earliest opportunity. Good laboratory practice (GLP) will be applied throughout the project in the acquisition, management and archiving of study data. In compliance with the ICH/GCP guidelines, regulations and in accordance with the University of Exeter Research Ethics, the Chief Investigator will maintain all records and documents regarding the conduct of the study. All data will be retained for a period of at

least five years after completion of the project. If the responsible investigator is no longer able to maintain the study records, a second person will be nominated to take over this responsibility.

10.4 Methods for data sharing

The sponsor and investigators are fully committed to share data in accordance with the HRA Policy. High volume data generation is not a feature of the proposed project, and so the data will be predominantly disseminated via publication in high-impact peer-reviewed journals (within 12 months of study completion) and via presentation at conferences. Publications will be in accordance with the University of Exeter's Open Access policy and where possible immediately made openly available to adhere to the funder's policy. All data published and/or shared will be anonymized to prevent subject identification. Importantly, on request we will make available our primary data to external researchers, subject to preservation of intellectual property and written acknowledgement that data ownership will reside with the research team. While it is not anticipated, data that is not included for publication but may be of value to the research community will be archived in the University's Institutional Repository, Open Research Exeter (ORE). All data will be deposited and will be citable using a persistent identifier.

10.5 Proprietary data

The results generated from this study will give insight in the role of fuel integration during inactivity, as well as identify potential molecular treatment targets which could be of interest to major Pharma companies who currently have exciting programs around preventing muscle wasting. The University of Exeter Innovation, Impact and Business (IIB) team can help with industrial liaison and knowledge transfer, from IP protection to finding a company interested in the research outputs.

11 ETHICAL AND REGULATORY CONSIDERATIONS

11.1 Research Ethics Committee (REC) review and reports

The study will be conducted in compliance with the principles of the Declaration of Helsinki (1996), the principles of GCP and in accordance with all applicable regulatory requirements. Local ethical approval was obtained from the University of Exeter's Sport and Health Sciences Ethics Committee (please see attached certificate of ethical approval). This application is now submitted for REC approval as part of the Clinical Research Network portfolio adoption process, so that the RD&E Pharmacy can be accessed for dispensing of the drugs.

Before the start of the study, approval will be recognised from a REC for the study protocol, informed consent forms and any other relevant documents. Substantial amendments that require review by REC will not be implemented until the REC grants a favourable opinion for the study. All correspondence with the REC will be retained in the Trial Master File. The Chief Investigator will submit an annual progress report (APR) to the REC within 30 days of the anniversary date on which the favourable opinion was given, and annually until the study is declared ended. The Chief Investigator will notify the REC of the end of the study, and if the study is ended prematurely, the Chief Investigator will notify the REC, including the reasons for the premature termination. Within one year after the end of the study, the Chief Investigator will submit a final report with the results, including any publications/abstracts, to the REC.

11.2 Peer review

The scientific quality of this research has been assessed within the research team and CI's host institution, before submitting this as part of the CI's application for a Sir Henry Wellcome Postdoctoral Fellowship. The Fellowship application process consisted of two rounds of written submissions (please find reviewers comments from 3 independent expert reviewers included in this application) and an interview round with the Wellcome Trust's Basic Science Panel, consisting of approximately twenty panel members from various UK institutions. Dr Marlou Dirks was awarded this 4-year Fellowship in November 2017.

11.3 Indemnity

The University of Exeter has expanded its standard indemnity cover with an extra medical malpractice extension, specifically for this study (please see documents attached). This ensures that Dr Rob Andrews, whose activity on this study is not covered by the NHS indemnity scheme, is fully covered in his role of medical collaborator. This insurance covers the potential harm to participants arising from the management, design, and conduct of the research.

12 DISSEMINIATION POLICY

The current proposal aims to conform to the principles of the 'European Charter for Researchers' by guaranteeing that the novel knowledge generated by the study is disseminated in a way that can be understood by the general public. The primary goal of these efforts is to distribute the key findings to the society and its affected individuals, as well as to health departments, policy makers, researchers, and health advocacy groups. The main mediums of communication and information dissemination are:

1) Peer-reviewed journal article(s). Data will be published in peer-reviewed journals according to RCUK/HEFCE guidelines, with gold or green access. The data will be published as one or more journal articles, depending on the outcomes of the study.
2) A project website that will be launched to inform the general public on the objectives, funding body, conclusions, and general recommendations of the project.
3) Social media such as specifically created Twitter and Facebook pages as well as my personal Twitter account. Data will also be shared via ResearchGate.com, where a project page for this study will be set up.
4) Local events. Examples of these are Soapbox Science, which showcases research of women who are making significant contributions to the scientific community, and Pint of Science, a festival which allows scientists to discuss their research with the general public in a local pub.

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