Study Title: The effects of repeated moderate overnight normobaric hypoxia on glucose homeostasis, appetite, body weight, inflammation and oxidative stress in individuals with type 2 diabetes mellitus.

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Investigators:	See protocol development team below
Sponsor:	University of Portsmouth
Funder (if applicable):	Leaders Development Institute
Signatures:	

Protocol Development Team:

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Co- investigator 2	**- Associate Prof Zoe Saynor**	**(University of Portsmouth)**
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Co- investigator 7	**- Prof Mike Tipton**	**(University of Portsmouth)**
Co- investigator 8	**- Prof Mike Grocott**	**(University of Southampton)**

Co-investigator 9 **- Prof Hugh Montgomery (University College London)**

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Co-investigator 13 **- Dr Janine Makaronidis (University College London)**

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1. AMENDMENT HISTORY

Amendment No.	Protocol Version No.	Date issued	Author(s) of changes	Details of Changes made
1	1.2	22-12-21	Dr A Shepherd	1) Added Dr Janine Makaronidis to the protocol team 2) Added details of stabilisers to blood collection tubes for specific analysis (i.e. incretins, ghrelin and leptin).
2	1.3	26-01-22	Dr A Shepherd	1) Added details on additional faecal analysis
3	1.4	16-03-22	Mr T James	1) Changed threshold for FEV1:FVC ratio lung function test in screening visit.
4	1.5	26-04-22	Mr T James	1) Added GP text messages for recruitment.
5	1.6	23-06-22	Mr T James	1) Increase recruitment capacity from 15 to 20 individuals.
6	1.7	14-07-22	Dr A Shepherd	1) Increase recruitment capacity from 20 to 23 individuals.

2. SYNOPSIS

Study Title	The effects of repeated moderate overnight normobaric hypoxia on glucose homeostasis, appetite, body weight, inflammation and oxidative stress in individuals with type 2 diabetes mellitus.
Internal ref. no.	n/a
Problem statement	The number of people with type 2 diabetes mellitus (T2DM) continuing to rise, this pandemic is expected to reach 700 million people by 2045. T2DM is a metabolic condition characterized by progressive insulin resistance and chronic hyperglycemia (high blood glucose concentrations). Hyperglycaemia increases the risk of both micro- and macrovascular damage, whilst interventions that reduce blood glucose mitigate this risk. Weight loss, achieved through exercise and dietary modification, is effective at reducing hyperglycaemia. However, despite the clear benefits of exercise and weight loss, diverse psychological, sociological and logistical factors can make it difficult for some individuals with T2DM to initiate, or adhere to, these lifestyle interventions. Alternative approaches to treatment are therefore required. The purpose of this research project is to investigate whether 10-days of overnight exposure to moderate hypoxia is effective at improving blood glucose control in individuals with T2DM and to provide insight into the physiological mechanisms responsible for any beneficial effects.
Research question / hypothesis	**Aims:** The primary aim of this research project is to investigate whether a 10-days of overnight moderate hypoxia can improve blood glucose control in individuals with T2DM. **Objectives:** Specifically, our objective is to assess a novel therapeutic intervention for the treatment and management of T2DM which overcomes many of the barriers to uptake and adherence that are associated with some lifestyle interventions such as exercise and weight loss. **Hypothesis** We hypothesise that 10-days of overnight moderate hypoxia will: 1. Improve glucose control 2. Improve insulin sensitivity 3. Reduce appetite / food intake 4. Reduce body mass 5. Improve inflammatory status 6. Improve redox balance 7. Increase gut anaerobe content 8. Maintain habitual physical activity 9. Have no effect on sleep quality

Study Design	Single blind, randomized, balanced, crossover design study (i.e. participants will undergo both the hypoxia and sham conditions and will be blinded to the conditions in which they are in).
Study Participants	Individuals with type 2 diabetes
Planned Sample Size	The study of 23 subjects provides adequate power (including a 15% drop-out rate typically seen in our clinical trials): 13 subjects are required to detect a 1 standard deviation change [30] in venous blood [glucose] during a 2 hour oral glucose tolerance test (OGTT (alpha = 0.05; power = 80%)).
Follow-up duration	1 months washout – 10 day intervention
Planned Study Period	1 year
Primary Objective	Post-intervention (sham vs hypoxia) area under curve for venous blood [glucose] during a 2 hour OGTT.
Secondary Objectives	Fasting venous blood [glucose], peak venous blood [glucose] during OGTT, Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), daily glucose excursions (continuous glucose monitoring), and untargeted metabolomics, body mass / composition change, hormonal markers of appetite (e.g. leptin and ghrelin), food intake, gut microbiome, markers of inflammatory status (e.g. interleukin-6; nitrite), redox balance (TNFα; SOD), HIF-1α, extracellular HSP70, physical activity and sleep quality.
Primary Endpoint	Post-intervention (sham vs hypoxia) area under curve for venous blood [glucose] during a 2 hour OGTT.
Secondary Endpoints	See secondary objectives.
Intervention (s)	10 days of moderate home-based overnight normobaric hypoxia (F_IO_2 = ~0.155, or sham hypoxia intervention (F_IO_2= 0.2093) using hypoxic 'pillow tents'.

3. ABBREVIATIONS

CHaRT	Clinical, Health and Rehabilitation Team
CRF	Clinical record folder
F_IO_2	Fraction of inspired oxygen
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
NHS	National Health Service
T2DM	Type 2 diabetes mellitus

4. BACKGROUND AND RATIONALE

Type 2 diabetes mellitus (T2DM) is a metabolic condition characterized by progressive insulin resistance and chronic hyperglycemia (high blood glucose concentrations) (10). Hyperglycaemia increases the risk of both micro- and macrovascular damage, whilst interventions that reduce blood glucose mitigate this risk (32). Weight loss, achieved through exercise and dietary modification, is effective at reducing hyperglycaemia (22). However, despite the clear benefits of exercise and weight loss, diverse psychological, sociological and logistical factors can make it difficult for some individuals with T2DM to initiate, or adhere to, these lifestyle interventions (1, 9). With the number of people with T2DM continuing to rise, this pandemic is expected to reach 700 million people by 2045 (24). Thus, there is a clear need for cost-effective interventions that can effectively improve glycaemic control in people with T2DM and which people will adhere to.

A simple exposure to a lowered concentration of inspired oxygen (i.e. hypoxia) may represent such an intervention. In addition to the beneficial effects on glucose homeostasis that have been reported following a single acute hypoxic exposure, repeated intermittent, or continuous, hypoxic exposure may also have therapeutic potential in individuals with T2DM. In rodent models, daily hypoxic exposures returned fasting blood [glucose] to normal levels and increased glucose transporter 4 translocation in mice with T2DM (6, 35). Similar effects on glucose homeostasis have been shown in overweight humans (17) and those with insulin resistance, (during intermittent hypoxic training) which was explained, at least in part, by reduction in body mass (~ 1.2 kg) (27).

Another mechanism for hypoxia induced weight loss could be a shift in the gut microbiome. The gut is a hypoxic environment and as the body becomes systemically hypoxic, anaerobes are likely to become more dominant (11). Some anaerobes are fermenters which not only produce alcohol but also short and medium chain fatty acids which can be metabolised (2, 25). Specifically, the hypoxia-inducible factor (HIF) pathway is attenuated with upregulation of the pyruvate kinase 1 and subsequent inhibition of pyruvate dehydrogenase (21). Moreover, the gene encoding PPAR is selected for during high altitude which leads to a shift in fatty acid

oxidation to glycolytic pathways (19) which in turn will lead to reduction in oxidative phosphorylation and thus oxygen demand, with more efficient oxygen utilisation.

Although hypoxia has received little attention in this kind of clinical context, sleeping in hypoxic environments is already common practice in competitive endurance athletes who attempt to gain an ergogenic benefit through up-regulating erythropoiesis (23). Our own recent work has also demonstrated a significant reduction in body mass in athletes following 10-days of overnight sleeping in a moderate normobaric hypoxic environment ($F_IO_2 = 0.156$) combined with moderate daily exercise in a hot environment (23), an effect which did not appear to be due to diuresis. The mechanisms underpinning the improved glycaemic control in response to hypoxia are likely multifactorial. Animal data and *in vitro* human skeletal muscle work (8) suggest that hypoxia may function as an exercise mimetic (i.e the physiological response to exercise without moving) by acting on the same (insulin independent) signalling pathway as that stimulated by muscle contraction, although improved insulin sensitivity in some studies (e.g. (18)) implies that insulin-dependent glucose transport may also have been favourably influenced. Hypoxic stress also activates the hypoxia inducible factor (HIF) pathway, which increases basal metabolic rate, suppresses appetite (20) and promotes a substrate shift towards glucose (15) (i.e. larger anaerobic contribution which is less efficient). HIF activation also induces the heat shock response and increases heat shock protein (HSP) expression which improves insulin resistance and glucose homeostasis in rodents and humans (16). Any reduction in appetite and associated body weight-loss is also likely to be important given that body mass reduction is linked to improved glycaemic control in individuals with T2DM (36).

In summary, T2DM is a condition that affects a significant proportion of the global population, with unsustainable health care implications. This project has the potential to deliver a novel, home based, non-invasive therapeutic intervention for individuals with T2DM. However, there is a dearth of evidence in people with T2DM to show the mechanisms for any beneficial effects. Importantly, the proposed intervention may overcome a number of barriers to uptake and adherence that are associated with these lifestyle interventions, which we anticipate will facilitate higher levels of uptake and compliance. As a consequence, this intervention has the potential to improve outcomes in individuals with T2DM, and to deliver significant financial

savings in terms of the clinical care costs associated with the treatment of type 2 diabetes mellitus.

5. PRELIMINARY STUDIES AND EXPERIENCE OF INVESTIGATORS

Dr Ant Shepherd's research focuses on using novel interventions (for example; extreme environments or nutrition) to improve metabolic and cardiovascular health, quality of life and physical function. Ultimately, his research revolves around patient benefit. In the past 3 years he has been the principal investigator or co-investigator on over £1,300,000 of funded projects related to understanding chronic diseases and/or assessing simple, low cost interventions which might improve health. He primarily works with individuals with type 2 diabetes mellitus (and different forms of insulin resistance) (3, 13, 26, 29, 31) but has also worked with a number of different clinical conditions (including obesity, end stage renal disease, cystic fibrosis, Raynaud's phenomenon, chronic obstructive pulmonary disease and stroke). He recently led a randomised controlled trial examining the effect of passive heating in individuals with type 2 diabetes (12). He is currently examining the effect of acute periods of hypoxia on appetite, substrate utilisation and glycaemic control in overweight and obese individuals.

Associate Prof Corbett's research examines the impact of environmental stressors (heat, cold and hypoxia) on performance, health and disease. His recent work has included a range of clinical groups including individuals with cystic fibrosis, type 2 diabetes, overweight and obesity. His recent work has shown the utility of an acute passive heating intervention in elevating heat shock protein in individuals with type 2 diabetes (12) important because decreased levels of heat shock protein correlate with insulin resistance. His current research addresses the role of chronic heat exposure in regulating heat shock protein expression, and glucose tolerance, in individuals with type 2 diabetes. Also recently, he has studied the utility of using oxygen saturation variability (entropy) (4) and network physiology approaches (14) to identify the negative sequelae of hypoxemia, and to develop non-invasive methods by which to assess the engagement of the respiratory control system in health and disease. He leads an ongoing program of work that builds from previous research demonstrating significant body mass reductions in athletes with 10 days of overnight hypoxic exposure (23), and is examining the role of hypoxia on appetite, substrate utilisation and glycaemic control in overweight and obese individuals.

Professor Cummings is Consultant Physician in Diabetes and Endocrinology at Portsmouth National Health Service Trust and Honorary Professor of Diabetes and Endocrinology at University of Portsmouth. He is Clinical Lead (Diabetes) for the Comprehensive Local Research Network for the National Institute for Health Research), Wessex. He is also Head of the DOVE (Dysglycaemia, Oxidative stress and the Vascular Endothelium) research programme and associate Editor for the journal *Practical Diabetes* and Sub-Editor for *Diabetes Digest*. His responsibilities will be to help with participant recruitment, screening of electrocardiograms (ECGs) and blood profiles. He will also help monitor the occurrence of adverse events.

Professor Tipton is a Professor of Human & Applied Physiology, director of our world leading Extreme Environments Laboratory at the University of Portsmouth and Editor in Chief of *Experimental Physiology* (a flagship journal of the physiological society). He is a world leader in extreme environmental physiology, and in the effect of hypoxia on human physiology. His responsibilities will involve helping with conceptualising the study, data interpretation and

reviewing of reports and manuscripts. He will act in a senior advisory role, and will mentor other members of the team.

Professor Montgomery is a Professor of Intensive Care Medicine at University College London, where he directs the Centre for Human Health and Performance. He is also a consultant in intensive care at the Whittington Hospital in North London. He has published over 500 papers, and has a 25-year history of studying the physiological response to hypoxia in sickness and in health. He was research lead for the 2007 Caudwell Xtreme Everest research programme. He has also run a large number of clinical investigations, and large scale randomised human trials. He has contributed to conceptualising the study and will assist in data monitoring, interpretation and analysis, and will review and co-author reports and manuscripts as may be sought.

Professor Grocott is Professor of Anaesthesia and Critical Care at the University of Southampton and Consultant in Critical Care Medicine at University Hospital Southampton NHS Foundation Trust. He leads the Critical Care Research Area within the Southampton NIHR Respiratory Biomedical Research Centre, is Head of the Integrative Physiology and Critical Illness Group at the University of Southampton and is Director of the Xtreme Everest Hypoxia Research Consortium. His responsibilities involve conceptualising the study, helping with data interpretation, reviewing of reports and manuscripts.

Associate Prof Zoe Saynor is a Senior Lecturer in Physical Activity, Exercise and Health at the University of Portsmouth and lead for the Physical Activity, Health and Rehabilitation Thematic Research Group and the Clinical, Health and Rehabilitation Team (CHaRT). She is also an honorary researcher at several NHS Trusts (Portsmouth Hospitals University NHS Trust; University Hospitals Southampton NHS Foundation Trust) and has a varied profile of national and international clinical research studies. She is experienced in the running of clinical trials and is an expert member of several clinical exercise committees, e.g. the European Cystic Fibrosis Society Exercise Working Group; UK Exercise and Lifestyle Clinical Studies Group as well as being a National Institute for Health Research (NIHR) Applied Research Collaboration (ARC) Wessex faculty member. Her responsibilities will involve helping with data interpretation, reviewing of reports and manuscripts.

Dr Rebecca Neal (Rendell) is a Senior Lecturer in Exercise Physiology and is an experienced environmental physiologist with extensive experience at employing home based hypoxic trials. Her responsibilities will involve helping with data interpretation, reviewing of reports and manuscripts. She will also help with the logistics of conducting home-based trials having published successful at-home hypoxic interventions using the proposed techniques.

Dr Heather Massey is a Senior Lecturer in Sport, Health and Exercise Science. Her main research interests are with in Environmental Physiology and has been IRMER trained, with recent experience of using the School of Sport, Health and Exercise Sciences DEXA scanning equipment. Her responsibilities will involve conducting and interpreting DEXA scans and reviewing reports and manuscripts.

Dr Maria Perissiou is a Lecturer in Clinical Exercise Physiology at the University of Portsmouth and is an experienced cardiovascular physiologist with experience in running

research trials in clinical population and people with T2DM. Her responsibilities will involve helping with data interpretation, reviewing of reports and manuscripts.

6. AIMS AND OBJECTIVES

Aims:

The primary aim of this research project is to investigate whether a 10-day overnight moderate hypoxia intervention can improve blood glucose control in individuals with T2DM.

Hypothesis
We hypothesise that 10-days of overnight moderate hypoxia will:

1. Improve glucose control
2. Improve insulin sensitivity
3. Reduce appetite / food intake
4. Reduce body mass
5. Improve inflammatory status
6. Improve redox balance
7. Increase gut anaerobe content

8. Maintain habitual physical activity
9. Have no effect on sleep quality

6.1 Primary Objective

Specifically, our objective is to assess a novel therapeutic intervention (hypoxia) on glucose AUC during an OGTT.

6.2 Secondary Objectives

Secondary objectives include fasting venous blood [glucose] peak venous blood [glucose] during OGTT, Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), daily glucose excursions (continuous glucose monitoring), body mass / composition change, gut microbiome, hormonal markers of appetite (e.g. leptin and ghrelin), food intake, markers of inflammatory status (e.g. interleukin-6; nitrite), redox balance (TNFa; SOD), HIF-1α, extracellular HSP70, physical activity and sleep quality (accelerometery).

7. STUDY DESIGN

7.1 Summary of Study Design

Single blind, randomized, balanced, crossover design study (i.e. participants will undergo both the hypoxia and sham conditions and will be blinded to the conditions in which they are in).

7.2 Primary and Secondary Endpoints/Outcome Measures

Primary outcome measure

Post-intervention (sham vs hypoxia) area under curve for venous blood [glucose] during a 2 hour OGTT.

Secondary outcome measures

Fasting venous blood [glucose] peak venous blood [glucose] during OGTT, Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), daily glucose excursions (continuous glucose monitoring), and untargeted metabolomics, body mass / composition change, hormonal markers of appetite (e.g. leptin and ghrelin), food intake, gut microbiome, markers of inflammatory status (e.g. interleukin-6; nitrite), redox balance (TNFα; SOD), HIF-1α, extracellular HSP70, physical activity and sleep quality.

8. STUDY PARTICIPANTS

8.1 Study Setting

The primary and secondary outcomes will be assessed in the Spinnaker building at the University of Portsmouth. The Clinical Exercise Physiology Laboratory at the University of Portsmouth has a successful history of conducting studies in clinical populations. The laboratory contains state of the art equipment that is required for conducting this study and a research group involving over 20 academics and researchers. Screening bloods will be assessed at Queen Alexandra Hospital, Portsmouth pathology lab or a commercial laboratory. Leptin, ghrelin, interleukin-6, TNFa, SOD, HIF-1α and extracellular HSP70 concentrations will be quantified using commercially available ELISA assays at UoP (or at UCL with Dr Janine Makaronidis). Profiling of the gut microbiome will be analysed between UoP (in Dr Sam Robson's Laboratory) and the University of Southampton. Nitrite will be analysed at Loughborough University using a Sievers nitric oxide analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK) with our established technique (5, 28-31). Metabolomics will be analysed at the University of Liverpool (in Professor Warwick Dunn's laboratory).

8.2 Overall Description of Study Participants

We will recruit 23 participants with T2DM as defined by the World Health Organization to take part in our simulated altitude exposure study (i.e. normobaric decreased FiO_2).

8.3 Eligibility Criteria

Note: Altitudes < 2,500m (~0.15 FIO2) are similar to those in a commercial aircraft and are considered safe for individuals with stable coronary artery disease (Bonadie et al., 2016).

Inclusion Criteria

1) Males and females with T2DM (as diagnosed with the WHO criteria).
2) Ability to understand the study and provide informed consent.

Exclusion Criteria

Individuals with contraindications to hypoxic exposure such as;

1) obstructive sleep apnoea,
2) extant cardiac conditions
3) on medications such as SGLT2 inhibitors or PPAR antagonists
4) glycated haemoglobin < 48
5) sickle anemia,
6) thalassemia,
7) haemoglobinopathy
8) FVC < 80% of predicted FVC
9) $FEV_{1:FVC\ ratio}$ < 5th percentile for age and sex (as according to the Eurpoean Repiratory Society and American Thoracic Society guidelines) (37) .

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9. SAMPLING

The study of 20 subjects provides adequate power (including a 15% drop-out rate typically seen in our clinical trials): 13 subjects are required to detect a 1 standard deviation change [30] in venous blood [glucose] during a 3 hour oral glucose tolerance test (OGTT (alpha = 0.05; power = 80%)). Seasonal changes will be recorded and corrected for as a covariate.

10. STUDY PROCEDURES

Consent, screening and familiarisation (visit 1): fam and hypoxic screen

Once participants have provided fully informed written consent, they will complete a health history questionnaire including additional questions specific to exercise in a hypoxic environment. This form will be signed off by the Principal Investigator following each participants' responses to the questionnaires to assess eligibility. If there are ANY questionable responses, these will be check by our independent medical officers (medcis whose expertise are in extreme environments) or our diabetes consultant (depending on the nature of the response). Further to this, all participants are required to undertake a spirometry tests and blood pressure assessment, provide a venous blood sample and have a 12 lead ECG carried out. Blood will be sent for analysis and screened for any health complications that may affect their participation in this study including, glycated hemoglobin, sickle anemia, thalassemia, and haemoglobinopathy. Results from the 12 lead ECG will be examined by a clinician as will any health history questionnaires that demonstrate questionable responses.

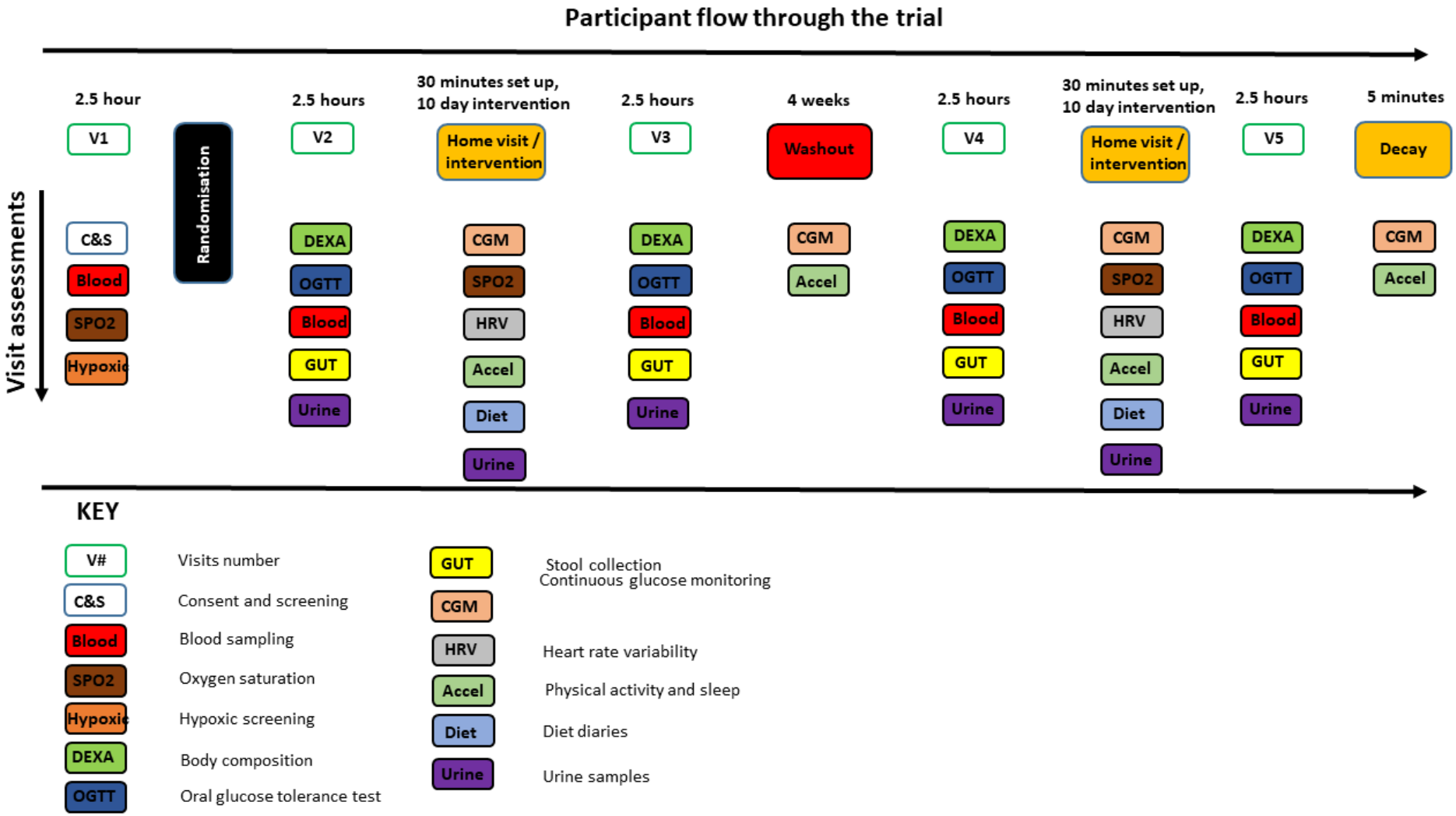
Hypoxic screening and familiarisation

Participants will be assessed for lung function (FVC and FEV_1/FVC ratio, PEF). Participants will undergo a one hour, supine exposure to hypoxia (F_IO_2 = ~0.155, adjusted based upon oxyhaemoglobin saturation during pilot hypoxic exposure to ensure target SpO_2 of ~88-92 %). Participants' desaturation will be recorded. Anyone who desaturates to below 65 % SpO_2 or in whom end tidal CO_2 or O_2 of less than 25 mmHg or 45 mmHg respectfully is recorded. Eligible participants will then be randomly allocated (using randomizer.org) to start in either the hypoxia or sham condition (see details below). Participants will be sent home with a SpO_2 (Equivital EQ02, Hidalgo, UK) monitor that will record oxygen saturation for 2 nights so that we can screen for undiagnosed sleep apnoea. Finally, a time will be scheduled within the next two days to set up the hypoxic generator, and the hood at the participant's home.

Intervention

Participants will undertake 10 days of moderate home-based overnight normobaric hypoxia (F_IO_2 = ~0.155), and a sham hypoxia intervention (F_IO_2= 0.2093) using hypoxic 'pillow tents' in a random order. The use of pillow tents rather than whole body tents was based on patient and public involvement (PPI) consultations. Participants will wear an SpO_2 monitor at all times within the tent that will sound an alarm if SpO_2 falls too low. If it sounds, participants will remove the hood (so that they breathe ambient air) and leave it off for the remainder of the night. An independent medical officer will decide if the participants can remain in the trial. The O_2 fraction will be set by the researcher and participants instructed not to change the settings. Finally participants will be asked to maintain a diary of the time spent in the tent. Finally, participants will be asked to record a dietary diary and photograph meals in order to try and replicate it in both conditions. Participants will then undertake the alternative condition. The cross-over conditions will be separated by a minimum 4 week wash out period. During the intervention, as well as recording safety information (ie. SpO_2) we will ask participants to wear a continuous glucose monitor (CGM) sensor (Abbott Diabetes Care Inc., Alameda, CA, USA) on the anterior portion of the upper arm with a small adhesive patch. These sensors will be worn for 14 days to determine free-living glycaemic control, in addition to a wrist-worn accelerometer (GENEActiv, Activinsights, Kimbolton, Cambridge, UK) to assess baseline habitual physical activity (PA) and sleep [32] (see details below in section 10.4).

Visit 2, 3, 4 and 5 (approx 2.5 per visit)
Visits 2-5 are identical in nature. Visits 2 and 4 are baseline visits before each arm of the crossover (i.e. the intervention). Visits 3 and 5 are following the 10 day hypoxic or sham exposures to assess the outcomes. An OGTT, body composition and venous blood samples and a stool collection will occur on each visit. After visit 3 there will be a 4 week washout out period. Visit 4 is necessary to ensure participants are back to baseline. During this visit, if anyone has had a respiratory tract infection or infectious disease in between visits, the hypoxic screening and lung function tests will be repeated. During visit 3, a new CGM sensor will be secured and accelerometer worn and a home visit for set-up will be re-booked. At the end of visit 5, another CGM and accelerometer will be affixed to the participants to check how long the effects of the intervention take to decay.
Urine collection is critical for our metabolomics analysis. In order to look for trends across time and between conditions we will need to collect urine before, during and after the intervention. Participants will be asked to collect a small urine sample for 2 days before the intervention starts, for each day of the intervention and for 2 days following the intervention. Participants will be provided with instructions on how to collect a mid-flow urine sample and how to safely store the samples until they are collected.


Participant flow through the trial
2.5 hour
V1
C&S
Blood
SPO2
Hypoxic
Randomisation
2.5 hours
V2
DEXA
OGTT
Blood
GUT
Urine
30 minutes set up, 10 day intervention
Home visit / intervention
CGM
SPO2
HRV
Accel
Diet
Urine
2.5 hours
V3
DEXA
OGTT
Blood
GUT
Urine
4 weeks
Washout
CGM
Accel
2.5 hours
V4
DEXA
OGTT
Blood
GUT
Urine
30 minutes set up, 10 day intervention
Home visit / intervention
CGM
SPO2
HRV
Accel
Diet
Urine
2.5 hours
V5
DEXA
OGTT
Blood
GUT
Urine
5 minutes
Decay
CGM
Accel
Visit assessments
KEY
V# Visits number
C&S Consent and screening
Blood Blood sampling
SPO2 Oxygen saturation
Hypoxic Hypoxic screening
DEXA Body composition
OGTT Oral glucose tolerance test
GUT Stool collection
CGM Continuous glucose monitoring
HRV Heart rate variability
Accel Physical activity and sleep
Diet Diet diaries
Urine Urine samples

10.1 Recruitment

Our typical recruitment rates via GP practices is approximately one in ten people with T2DM. GP recruitment will be executed via mail outs and text messages (e.g. We are looking for people with type 2 diabetes to sleep at home in slightly reduced oxygen for 10 nights which may help manage their diabetes and help them lose weight - click the link to find out more). Recruitment via the local DESMOND course (T2DM education clinic) is approximately one in five people with T2DM. We will need to approach between 90 and 113 people with T2DM to ensure we recruit enough (n = 23) people to account for drop outs.

10.2 Screening and Enrolment

For word of mouth recruits, the research team will provide interested parties with an invitation letter and the PIS. Our local GP collaborators will send our PIS and a letter which will include a free post return slip for potential participants who are interested. A member of the UoP research team will attend the DESMOND meeting 3 times a month to advertise, discuss and disperse information about the study.

For actual participant screening following consent, please see part 10 (one page above).

10.3 Randomisation

Eligible participants will be randomly allocated (using randomizer.org) to start in either the hypoxia or sham condition (see details below).

10.4 Study Assessments

OGTT

Fasted blood samples will then be taken for the measurement of plasma glucose and insulin concentrations ([glucose] and [insulin], respectively), and these data will be used to calculate β-cell function via the HOMA-%B model. Following this, participants will consume 75 g of glucose, and blood samples will be taken at 0 and 120 minutes post-glucose ingestion for the assessment of plasma [glucose] and [insulin]. Utilising the data from the OGTT protocol, insulin sensitivity of those not on insulin will be estimated using Stumvoll's insulin sensitivity index [31].

Dual-energy X-ray absorptiometry (DEXA) scanner

The scan will be carried out using a DEXA scanner (Hologic, Vertec, UK) operated by a trained and qualified researcher (Dr Heather Massey). A whole body scan will be conducted on each participant and a post-collection segmental analysis will be conducted afterwards. Variables that will be calculated from the scan data are fat density, muscle density and bone density; the scan will produce a density value measured in $g.cm^{-3}$. This will give us a baseline analysis of body composition and will allow us to identify if the expected weight loss of the intervention is fat or fat free mass.

Continuous glucose monitoring (CGM)

The sensors (Abbott Diabetes Care Inc., Alameda, CA, USA) will be fixed to the anterior portion of the upper arm with a small adhesive patch. These sensors will be worn for 14 days to determine free-living glycaemic control.

Habitual Physical Activity and Sleep (accelerometry)

Physical activity (PA) and sleep will be assessed using wrist-worn accelerometers (GENEActiv, Activinsights, Kimbolton, Cambridge, UK). These devices have previously been validated for use in healthy adult populations (7) and are extensively used in clinical studies. The GENEActiv accelerometers will measure triaxial movement acceleration in gravity (g) units (1 g = 9.81 m/s^2) at a frequency of 100Hz continuously over a period of 7 days. The Euclidean norm (magnitude) of signals from the three axes minus 1 g (with negative numbers rounded zero) will be used to quantify acceleration due to movement in mg (1 mg = 0.00981 m/s^2) (33). Following the measurement periods, data will be downloaded using manufacturers software and processed in R (R Core Team, Vienna, Austria) using the open source GGIR software package (http://cran.r-project.org) following completion of the study. Previously validated acceleration threshold values (in healthy adults) will be used to quantify the time (minutes/day) spent on average in each intensity category: total PA, and separately for light, moderate, vigorous intensities and the composite category moderate-vigorous PA (MVPA). To facilitate comparison with current national PA guidelines PA, average MVPA accumulated in bouts of at least 10 minutes will also be calculated.

Sleep variables will be determined from accelerometer data using an open source sleep detection algorithm in GGIR software. Sleep metrics derived using this method have demonstrated good levels of agreement with both self-report measures of sleep and polysomnography (the gold standard). The method of accelerometer based sleep quantification used in this study is described in detail elsewhere (34). Briefly, wrist-worn triaxial accelerometers allow approximation of the angle of orientation of the arm relative to the horizontal plane. Periods of sleep are defined as nocturnal periods characterised by minimal movement frequency and magnitude of changes to the angle of the arm which does not include day time sleep. Time in bed will be defined as the onset of the first period of sustained inactivity (as measured by changes of less than 5 degrees in a rolling 5 minute window) to the end of the last period of inactivity. Sleep duration is the sum of all recorded periods of sleep. Sleep efficiency can then be calculated as the sleep duration as a proportion of time in bed.

Periods of accelerometer non-wearing will be identified using the range and standard deviation (SD) of acceleration values at each axis, calculated for rolling 60 minute windows. Non-wearing will be indicated if the SD is <13.0 mg or if the range of values is <50 mg for two of the three axes. A full explanation of this method can be found elsewhere (33). To allow effective assessment of habitual PA and sleep, measurement days when the accelerometer is worn for less than 16 hours will be excluded from the final analyses (33). Participants who recorded less than 4 days of 16 hours will also be excluded.

Oxygen saturation

An SpO_2 monitor (Equivital EQ02, Hidalgo, UK) will be set up at the participant's home. Participants will wear the monitor at all times during exposure to hypoxia. If saturation falls below (65%) an alarm will be sounded. This system also records beat to beat heart rate and will store the data on the device until it is collected. Participants will also be asked to maintain a diary of the time spent in the tent.

Diet diary

Participants will be asked to record a dietary diary and photograph meals for all 10 days of the intervention in order to try and replicate it in both conditions. This is an important step to enable us to assess any changes in the gut microbiome. We appreciate that this is a significant participant burden and it will be discussed in the section below.

Biochemistry

Fasting venous blood [glucose] peak venous blood [glucose] during OGTT, Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), hormonal markers of appetite (e.g. incretins, leptin and ghrelin*), food intake, markers of inflammatory status (e.g. interleukin-6; nitrite), redox balance (TNFa; SOD), HIF-1α and extracellular HSP70. We will also include an untargeted metabolomics strategy using two ultra performance liquid chromatography-mass spectrometry assays to measure the relative concentration of up to 2000 metabolites (plasma and urine). * For analysis of gut hormones, sample collection tubes need to be treated in specific ways. For incretin analysis (i.e. GLP-1 and PYY) we will prime the EDTA tubes with a 10 µl of DDP-IV inhibitor per mL of whole blood and 100 µl of aprotinin. The tubes are chilled on ice, and remain on ice until centrifuged. For analysis of leptin and Desacyl-Ghrelin 100 µl of aprotinin is added to an EDTA tube and for Acyl-Ghrelin and additional 20 µl of Pefabloc is added. Following centrifugation, both Ghrelin samples will be treated again (475µl of plasma to 25µl of Pefabloc).

Gut microbiota

Stool samples will be frozen at -80°C within 12 hours of collection. Bacterial DNA will be extracted using the QIAamp PowerFecal Pro DNA kit according to the manufacturer's instructions (51804, Qiagen, USA) at the end of data collection. The purified extracted DNA will be frozen at -80°C. The extracted DNA will be delivered to the University of Southampton, National Oceanography Centre, for PCR amplification of the 300 bp reads, V3-4 region of the 16S rRNA gene. DNA samples will be depleted for host DNA and enriched for bacterial DNA using primers spanning the full-length prokaryote-specific 16S rRNA amplicon. In this way, host DNA from the individuals will not be analysed, preventing any issues with personal identification. 16S rRNA sequencing libraries will be generated for the Illumina MiSeq sequencer. Sequenced 16S rDNA sequences will be compared against the SILVA database of known 16S rDNA sequences for taxonomic classification.

Comparisons of the gut microbiome will be made using the Alpha (α) diversity levels on basis of the observed diversity, Chao1 diversity and Shannon diversity metrics. Variability between the cohorts will be identified based on principal coordinates analysis. Significantly differentially abundant taxa will be identified between each cohorts' sample based on the use of generalised linear models using the DESeq2 package in R, using a fold change threshold of

2-fold difference between the cohorts with a p value (adjusted for multiple testing using Benjamini and Hochberg correction) <0.05.

Faecal samples will also be analysed (in collaboration with UCL) for short-chain fatty acids (acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, hexanoate), alcohols (ethanol, 1-propanol, 2-propanol, butanol, isobutanol, pentanol) and indoles (bacterial metabolites of tryptophan that have immunomodulatory effects).

10.5 Discontinuation/Withdrawal of Participants from Study Treatment

Participants may be withdrawn from treatment, or from the study as a whole; if they ask to withdraw, if participant safety is in question or if the study is stopped early.

10.6 Definition of End of Study

The end of study will be the date of the last visit of the last participant.

11. INTERVENTIONS

11.1 Description of Study Intervention / Treatment

10 days of moderate home-based overnight normobaric hypoxia (F_IO_2 = ~0.155, or sham hypoxia intervention (F_IO_2= 0.2093) using hypoxic 'pillow tents'.

11.2 Adherence to Study Treatment

Participants will be asked to confirm compliance and complete a diary confirming the exposure. SpO_2 will be recorded and used as confirmation.

11.3 Accountability of the Study Treatment

Ultimately, the chief investigator (Dr Ant Shepherd) is responsible for all aspects of the study. However, the appointed post-doctoral researcher will be responsible for installing the hypoxic generators and monitoring adherence.

11.4 Concomitant Medication / Therapies

Participant involvement is anticipated to be ~2 months. Medication will be recorded on enrolment and updated throughout their time in the study.

12. ASSESSMENT OF SAFETY (IF APPLICABLE)

A serious adverse event is any untoward medical occurrence that:

· Results in death,

· Is life-threatening,

NOTE: The term "life-threatening" in the definition of "serious" refers to an event in which the participant was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

· Requires inpatient hospitalisation or prolongation of existing hospitalisation,

· Results in persistent or significant disability/incapacity, or

· Is a congenital anomaly/birth defect.

· Other important medical events*

*Other events that may not result in death, are not life threatening, or do not require hospitalisation, may be considered a serious adverse event when, based upon appropriate medical judgement, the event may jeopardise the participant and may require medical or surgical intervention to prevent one of the outcomes listed above.

12.2 Reporting Procedures for Serious Adverse Events

A serious adverse event (SAE) occurring to participant should be reported to the REC that gave a favourable opinion of the study where in the opinion of the Chief Investigator the event was: 'related' – that is, it resulted from administration of any of the research procedures; and 'unexpected' – that is, the type of event is not listed in the protocol as an expected occurrence. Reports of related and unexpected SAEs should be submitted within 15 days of the Chief Investigator becoming aware of the event, using the NRES report of serious adverse event form (see NRES website).

12.3 Recording and Reporting Procedures for All Adverse Events

All adverse events will be recorded in participant file notes. Distinction between SAE and AE will be made by an independent medical officer. All SAE will be reported to the sponsor and the REC regardless of if it is related or not.

13. DATA HANDLING AND RECORD KEEPING

13.1 Data Collection Forms

Visit 1 will act as a consent, screening and hypoxic screening to allow for medical clearance. All data for these visits will be stored in the clinical record folder (CRF). Separate forms will be prepared for; anthropometrics, medical history, screening (i.e. blood pressures etc) and blood markers. All paper copies will be stored in the CRF's.

13.2 Data Management

All study data (excluding electronic files) will be entered on paper copies and stored in participant CRF's. Electronic files will be stored on a shared (with access restricted to only the research team) google drive. All data will be double data entered into excel. Macro's will be used to check for anomalies and corrected. The participants will be identified by a study specific participants number and/or code in any database. The name and any other identifying detail will NOT be included in any study data electronic file. The chief investigator (Dr Ant Shepherd) is responsible for database maintenance and management.

14. DATA ANALYSIS

14.1 Description of Analysis Populations

All participants who complete all visits will be entered into the analysis as per protocol.

14.2 Analysis of Endpoints

All data will be tested for normality using the Shapiro-Wilk test. For normally distributed data, paired samples t-tests will be used to assess differences pre and post hypoxic exposures for; blood [glucose] (AUC and peak), insulin sensitivity, eHSP70, IL-6, leptin, ghrelin, TNFa, SOD, HIF-1α HR, PA and sleep efficiency. For non-parametric data Wilcoxon-Signed rank test will be used for pairwise comparisons. Repeated measures one-way analysis of variance (ANOVA) will be used to identify differences between conditions for SpO_2 and [glucose] across time from the CGMs. Mauchly's test of sphericity will used to determine if any data violated the assumption of spherical data. If violated, sphericity will be accounted for using the Greehouse-Geisser correction. Where significant differences are observed ($P < 0.05$), post-hoc tests will be performed using LSD pairwise comparisons. Effect sizes will be reported as partial eta squared (η^2_p) (small = 0.01; medium = 0.06; large = 0.14) [33].

14.3 Procedure for Dealing with Missing, Unused and Spurious Data

Outliers will remain within the data. Within the data analysis software missing data will be coded 9999 and the data point missed during analysis.

14.4 Procedures for Reporting any Deviation(s) from the Original Statistical Analysis Plan

For this pilot a SAP is not required (excluding the above).

14.5 Interim analysis and criteria for early study termination

Given the small samples size and time frame, no interim analysis will be performed. Early termination will only occur if the safety of participants is in question.

15. THICS

NHS Local Research Ethics Committee (REC) approval will be obtained prior to commencement of the study. The REC, local NHS, research and development department and all site specific forms and patient identification centre forms will be forwarded to the funder prior to recruitment of participants. Written informed consent will be obtained from all participants. Insurance indemnity will be provided by the Sponsor for this study.

Every effort has been made to keep the risks and discomforts to a minimum but there are some risks associated to taking part. Not all volunteers will experience any or all of the risks stated below, but participants will be made aware of them.

1. The main burden to you will be a lifestyle alteration because you will be asked to sleep in a tent for 2, 10 day periods whilst wear smart monitors. During these periods you will also be collecting urine samples and recording your food consumption in diaries.
2. We will also take small blood samples which can cause pain for a short period. Likewise, putting the glucose monitors on can cause mild discomfit.
3. The body composition scan involves a mild dose of radiation. All scans in this study (per participant) would be equivalent to 3 day of natural background radiation. It is, however, the most accurate and appropriate way of assessing the changes in your body across the intervention.

15.1 Participant Confidentiality

The study staff will ensure that the participants' anonymity is maintained. The participants will be identified only by initials and a participants ID number on the CRF and any electronic database. All documents will be stored securely and only accessible by study staff and authorised personnel. The study will comply with the Data Protection Act which requires data to be anonymised as soon as it is practical to do so.

15.2 Other Ethical Considerations

N/A

15.3 Declaration of Helsinki

The study protocol will be carried out in accordance with the declaration of Helsinki.

15.4 ICH Guidelines for Good Clinical Practice

All staff will be GCP trained and will be monitored by the sponsor to ensure adherence to GCP.

16. PATIENT PUBLIC INVOLVEMENT (PPI)

16.1 Study design

Evidence of patient, carer and public involvement (PCPI):
The chief investigator has a strong track record of PCPI work having been involved in PCPI award winning projects from the UK Stroke Forum (2016) for an acceptability and feasibility trial. We have a PCPI co-applicant (Mrs Janet Rennell-Smyth) who has been instrumental in the design and development of this study and will continue to be involved in the reporting, dissemination and future NIHR funding bids. Other individuals with T2DM have been consulted and have stated that they would prefer hoods rather than full tent coverings. Questions surrounding the smart technology were made following reviewing the PIS and pictures of the technology have been added. The lay summary and patient facing documentation has been reviewed by our PCPI co-applicant.

16.2 Study implementation

This is a pilot study and as such we do not have a TSC or DMC. Issues will be managed by the senior members of the research team. We will endeavor to involve the PCPI member where possible on decisions regarding the study and data.

16.3 Dissemination

Our PCPI member will help disseminate findings through local groups, local/national media, and co-authoring the paper. Our PCPI involvement has developed the protocol to this stage and will continue to play a large role in this project and the larger definitive trial to come.

17. FINANCING AND INSURANCE

Total funds are **$120,000**. The full economic cost for this study is **$170,166**. Public liability and indemnity insurance is covered by the University of Portsmouth.

17.1 Research Costs

Total cost ($120,000)

Total funds funded are **$120,000**. In order to keep the cost within the maximum budget the University of Portsmouth have only requested 11% *($13,013)* of the available 15% overhead costs. The full economic cost for this study is **$170166**. Please see table 1 for the overview and text below for the detailed justification.

Table 1. Shows the breakdown of the study costs.

Breakdown of costs	**Full economic costing ($)**	**Funded ($)**
Research Associate 0.7fte	41402	41402
Recruitment	725	725
Consumables and Analysis	40933	40933
Small equipment	12843	12843
Participant travel	653	653
Publication Costs	2175	2175
PI A Shepherd 2 hrs p/w (88 hrs)	5406	5388
CoI Z Saynor 0.34 hrs p/w (2 days)	922	919
CoI J Corbett 0.34 hrs p/w (2 days)	1103	1099
CoI M Tipton 0.17 hrs p/w (1 day)	853	850
Infrastructure technician	2024	0
Estates Costs	15408	0
Indirect Costs	45719	13013

Total ($)	**170166**	**120000**

$ displayed are USD.

Value added to the project

The Extreme Environments Laboratory at the University of Portsmouth has a 30 year history of conducting innovative human research trials. The laboratory contains some of the world's leading facilities for conducting environmental research including three bespoke environmental chambers, with temperature, humidity, solar, wind and hypoxia control, and a research group involving over 20 academics and researchers. The Institute of Biological and Biomedical Sciences at the University of Portsmouth has state of the art laboratories to enable us to analyse our biological samples. As well as the bespoke chambers, it hosts portable kit essential for this study such as two hypoxic generators (cloud 9), SpO_2 monitors (Equivital EQ02, Hidalgo, UK) and accelerometers.

Researcher time (total = $80,536)

We request funds for an experienced researcher (senior research associate) in order to achieve the timeline goals (12 months, full-time = $71,555). They will assist with the day to day running of the project, including participant recruitment, study logistics, data collection, inputting and analysis (including biochemistry). We will need $725 for recruitment purposes (to ensure we get the correct candidate and advertise to the largest audience). Academic staff time for this grant totals $8,256.

Equipment (total = $12,843)

We will buy two hypoxic systems (cloud 9), and individual pillow tents (tents are not transferable between individuals for hygiene reasons and are considered a consumable) from Sporting Edge UK Ltd. These units will ensure we can complete this project in the time scale of the grant. Total = $12,843

Consumables (total = $12,956)

We require $12,956 for consumables so that we can take perform the experiment. Oral glucose tolerance tests, blood sampling kits and ELISA kits to analyse the hormones and proteins within the blood (including: insulin, HIF, HSP70, nitrate, nitrite, ghrelin, leptin, IL6, IL10, SOD and TNFa). Continuous glucose monitors will also be utilised to assess glucose concentrations. We have access to GENEActiv accelerometers, as such we are not asking for funds.

Participant travel (total = $652)

Our patient and public involvement group (PPI) were clear that funds for travel needed to be supported. In line with our other trials we will provide £10 per visit to cover the costs of transport (£10 x 3 visits x 15 people = £450 or $652).

17.2 Service Support Costs

17.3 Excess Treatment Costs

17.4 Study Sponsorship

This study is being sponsored by the UoP.

18. TIMETABLE AND ORGANISATIONAL CHART

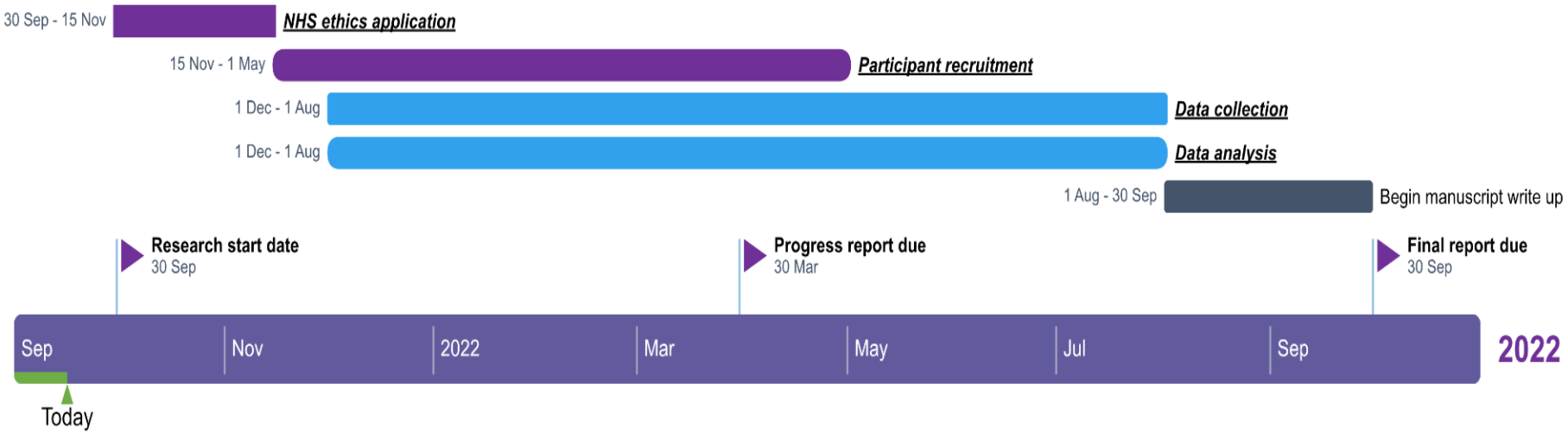


Figure 1 shows the suggested timeline for the project.

19. RESOURCES, EQUIPMENT AND PHYSICAL FACILITIES

The Extreme Environments Laboratory at the University of Portsmouth has a 30 year history of conducting innovative human research trials. The laboratory contains some of the world's leading facilities for conducting environmental research including three bespoke environmental chambers, with temperature, humidity, solar, wind and hypoxia control, and a research group involving over 20 academics and researchers. The Institute of Biological and Biomedical Sciences at the University of Portsmouth has state of the art laboratories to enable us to analyse our biological samples. As well as the bespoke chambers, it hosts portable kit essential for this study such as two hypoxic generators (cloud 9), SpO_2 monitors (Equivital EQ02, Hidalgo, UK) and accelerometers.

20. DISSEMINATION AND OUTCOME

Results (estimates of effect sizes and confidence intervals) from this pilot study will be utilised to define our primary outcome and to power a larger, definitive multi-centre randomised control trial with our co-applicants and collaborators around Europe. Once our final report has been submitted to the funder, we will apply for NIHR funding via a researcher-led Research for Patient Benefit (RfPB) or an Efficacy and Mechanism Evaluation (EME) bid (depending on the effect size and sample size). The results will be disseminated through publication in peer-reviewed scientific and clinical journals and via presentations, local, national and international meetings. A summary of the results will be sent to all the participants. Finally, an open day and presentation will be carried out where the participants will be informed of the study findings and have the opportunity to ask any pertinent questions and/or provide feedback.

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