Study Title: The Effect of Chronic Passive Heating on Insulin Sensitivity and Cardiovascular function in People with Type 2 Diabetes Mellitus: A pre- and post-test trial.

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1. AMENDMENT HISTORY

Amendment No.	Protocol Version No.	Date issued	Author(s) of changes	Details of Changes made
1	2	18/02/2021	Mr Thomas James	• Amendment to inclusion and exclusion criteria • Further detail of informed consent process added • Further detail on safety SOP's added
2	3	10/05/2021	Mr Thomas James	• Wording change of "nurses" to "UoP staff" • Identification of where screening blood samples will be analysed added.

2. SYNOPSIS

Study Title	The Effect of Chronic Passive Heating on Insulin Sensitivity and Cardiovascular function in People with Type 2 Diabetes Mellitus: A pre- and post-test trial.
Internal ref. no.	n/a
Problem statement	Type 2 diabetes mellitus (T2DM) is a metabolic condition characterized by chronic hyperglycemia and progressive insulin resistance, which progressively lead to macro- and microvascular damage and subsequent impairments in blood pressure (BP) control. Therapeutic approaches to manage T2DM focus on improving glycaemic control and BP and include pharmaceutical treatments (e.g. Metformin and insulin), physical activity and exercise, and calorie restriction. However, pharmaceutical interventions can be expensive and are associated with low adherence. Although exercise and diet programs have been shown to be effective, like pharmaceutical interventions, they often have poor adherence in people with T2DM. With the number of people with T2DM (464 million) continuing to rise and expected to reach 700 million by 2045, the costs associated with the clinical management of this condition are likely to become unsustainable. There is, therefore, a need to explore the potential of alternative interventions. In particular, interventions which may be cheaper than clinical management and have better adherence than exercise, and hypoglycemic agents, to improve glycemic control and deleterious cardiovascular manifestations of this condition.

	Passive heating may be one such intervention with therapeutic potential.
Research question / hypothesis	Before passive heating can be considered as a therapeutic option for improving metabolic and cardiovascular health in individuals with T2DM, we need to explore whether their chronic passive heating (1h x 10 days) is able to improve: 1) insulin sensitivity 2) glycaemic control 3) metabolic efficiency 4) vascular health
Study Design	We plan to run a within-subject, pre-, post-intervention study
Study Participants	Adults ≥ 35 years (post-menopausal for females), diagnosed with T2DM as defined by the world health organisation (WHO).
Planned Sample Size	We aim to recruit 20 individuals with T2DM.
Follow-up duration	7 days
Planned Study Period	18 months
Primary Objective	To determine whether chronic passive, warm water therapy increased insulin sensitivity in individuals with T2DM?
Secondary Objectives	To determine whether chronic passive, warm water therapy improves: 1) glycaemic control 2) metabolic efficiency 3) vascular health

Primary Endpoint	Improvement in insulin sensitivity from chronic passive heating.
Secondary Endpoints	1) fasting plasma [glucose] 2) AUC plasma [glucose] 3) peak plasma [glucose] 4) HbA_{1c} 5) plasma [eHSP70] 6) plasma [IL-6] 7) plasma [IL-10] 8) plasma [butyrate] 9) plasma [growth hormone] 10) RMR 11) fat oxidation 12) CHO oxidation 13) macrovascular function 14) microvascular function 15) resting heart rate 16) stroke volume 17) total peripheral resistance

	18) cardiac output 19) heart rate variability
Intervention (s)	8-12 x1 h water immersion (to the clavicle, @40 °C, rectal temperature ~38.5 °C and <39 °C) sessions over a period of 14 days.

3. ABBREVIATIONS

AE	Adverse event
ANOVA	Analysis of variance
AUC	Area under the curve
CHO	Carbohydrate
CPET	Cardiopulmonary exercise test
CRF	Clinical records file
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
eHSP	Extracellular heat shock protein
GCP	Good clinical practise
GH	Growth hormone (human)
Hb	Haemoglobin
$HbA1_c$	Haemoglobin adult type 1_c (Glycated haemoglobin)
Hct	Haematocrit
HEC	Hyperinsulinaemic-euglycaemic clap
HIIT	High intensity interval training
HR	Heart rate
HRV	Heart rate variability
HSP	Heat shock protein
IL	Interleukin
LF	Low frequency
NHS	National Health Service
NIHR	National institute for health research
NIRS	Near infrared spectroscopy
NRES	National research ethics service
OGTT	Oral glucose tolerance test
PCPI	Patient, carer and public involvement
PPI	Patient public involvement
QUICKI	Quantitative insulin sensitivity check index

REC	Research ethics committee
RHR	Resting heart rate
RMR	Resting metabolic rate
RMSSD	Root mean square of the successive differences
RSA	Respiratory sinus arrhythmia
SAE	Serious adverse event
T2DM	Type 2 diabetes mellitus
T_{rec}	Rectal temperature
UoP	University of Portsmouth
$\dot{V}O_2$	Oxygen consumption
WHO	World health organisation

4. BACKGROUND AND RATIONALE

Current estimates suggest 422 million people worldwide live with a form of diabetes, of which ~ 90% have type 2 diabetes mellitus (T2DM) [1]. The total direct and indirect cost of care for individuals with diabetes in the UK is £23.7 billion, equating to ~ 20% of the annual NHS budget [2], this figure is expected to rise to ~£39.8 billion by the year 2035 [2, 3]. Approximately 85-90% of cases of T2DM arise from a poor lifestyle and obesity [4-6], with the remaining 10-15% resulting from genetic predispositions [7, 8]. Current interventions include pharmaceutical treatments, exercise and calorie restrictive diets, which aim to improve glycaemic control. However, pharmaceutical interventions carry a high financial cost [9, 10], while exercise and diet programmes have a low adherence rate in individuals with T2DM [10-15]. With the prevalence of T2DM continuing to increase, the costs associated with the clinical care of these individuals are likely to become unsustainable. Simple, inexpensive interventions to improve the clinical profile of this group are therefore needed.

One emerging potential therapy to improve glucose homoeostasis is passive heating. Preliminary evidence suggests passive heating may have beneficial effects for metabolic health in animal models [16-21] and in humans [17, 22-24]. In 1999, the use of hot tubs (38-41°C , 30 mins / day for 3 weeks) was shown to reduce fasting plasma glucose concentrations by ~14% (1.3 $mmol.L^{-1}$) and decrease $HbA1_c$ by ~10-11 mmol/mol in 8 individuals with T2DM [23]. Given the rate of turnover in haemoglobin this reduction is surprising as the treatment period was run over 3 weeks and the total haemoglobin turnover takes ~115 days [25]. While more recent work has been conducted into the effects of a single bout of passive heating in healthy adults [22] and individuals with T2DM (including ourselves, under review) [22, 24], none have been done on chronic heating since Hooper [23]. Hooper [23] postulated that an increase in skeletal muscle blood flow may be responsible for this increased glucose clearance, citing evidence that this can modulate insulin mediated glucose uptake [26]. Other mechanisms have also been purported, but have yet to be elucidated, including; increased insulin sensitivity [27, 28], altered inflammatory response [29-31], activation of heat shock proteins (HSP) [32], altered gut microbiome and butyrate [33]. Repeated passive heating results in transient increases in deep body temperature [24, 34-37] and may improve glucose homeostasis [22, 38-46] via similar mechanisms to exercise.

Regular aerobic exercise also improves macro- [47] and microvascular function [47], muscle oxygenation [48], autonomic fuction [49], cardiorespiratory fitness [50], lung function [51] and delays age related muscle loss [52]. Acute exercise studies show that insulin sensitivity after 1h of moderate exercise does not change [53, 54], however, insulin sensitivity appears to be improved following bouts longer than an hour [54] or performed at greater intensity [55]. Increases in insulin sensitivity have a curvilinear relationship with energy expenditure [54] and could also be due to greater HSP expression [56]. However, it is unrealistic for people with T2DM to perform this level of activity. Passive heating may be one supplemental exercise

mimetic to augment improvements in insulin sensitivity, cardiorespiratory fitness [60] and muscle strength, and function [81].

We recently provided evidence that acute passive heating in poeple with T2DM (currently under review for publication) is well tolerated and increases extracellualr [HSP70], and energy expenditure, and reduce diabstolic blood pressure. There is a growing body of evdience that suggests passive heating may improve many facets of human physiology, however, the mechanisms that underpin these benefits have yet to be established and future research needs to explore these further.

5. PRELIMINARY STUDIES AND EXPERIENCE OF INVESTIGATORS

Dr Anthony Shepherd is the lead applicant and has expertise in trial management and clinical exercise physiology and his work has focused on exercise, (oxygen uptake, quality of life, functional capacity and glucose homeostasis) in clinical cohorts. Mr Thomas James is a PhD candidate currently working with a background in clinical, exercise and environmental physiology. He has recently completed a similar study in individuals with T2DM. Dr Jo Corbett is experienced in running exercise and thermal physiology trials and is a World leader in the area. Dr Maria Perissiou is an early career researcher with extensive experience in assessing the acute cardiovascular responses to exercise in clinical population. Dr Perissiou has expertise in exercise testing, assessments of cardiovascular function and venous cannulation in clinical populations. Dr Clare Elgin is an experienced thermal physiologist and has experience leading MoD trials. Prof. Michael Tipton's World leading expertise in thermal physiology will be used to help with data interpretation. Dr John Young's expertise in biochemistry will be used for data analysis and interpretation. Dr Zoe Saynor has expertise in exercise testing and training in clinical populations, venepuncture and cannulation and will help with data interpretation. Prof Michael Cummings (clinical lead for the local NIHR diabetes research network), will be involved in this project and will provide clinical advice, responsible for participant safety and helping with participant recruitment. Ms Sharon Allard and Dr Kathryn Carey-Jones will aid with participant recruitment through Clinics and primary care. Mr Harvey Belcher and Mr Dan Piccolo will help with data collection. Final report and project related publications will be a collaborative effort of the team.

6. AIMS AND OBJECTIVES

This study will provide us with important data that will help ascertain whether passive heating has the potential to be used as a future treatment for T2DM and also provide mechanistic insight to how passive heating effects individuals with T2DM.

6.1 Primary Objective

To determine whether chronic passive heating can increase insulin sensitivity in individuals with T2DM.

6.2 Secondary Objectives

To establish if chronic passive heating improves:

1) glycaemic control
2) metabolic efficiency
3) vascular health

7. STUDY DESIGN

7.1 Summary of study design

Plan of investigation: We plan to run a pre-, post-intervention study to assess chronic heating's effect on metabolic health and cardiovascular function in people with T2DM. We aim to recruit 20 individuals with T2DM. All visits (11-15) will take place within our physiology laboratories (Spinnaker Building, University of Portsmouth). The experimental design will involve an initial screening visit (~1 h), two identical experimental visits (pre and post intervention) (~ 3 h) and 8-12 intervention visits (~1.5 h) (see Figure 1). We will aim for 10 intervention visits [57], however for feasibility we have incorporated scope for plus or minus 2 immersions for individuals who want to come for extra visits or struggle with time constraints. Both longer [58] and shorter protocols [59] have been utilized in the past. The screening visit will consist of participant characterisation, safety assessments including resting electrocardiogram (ECG), screening bloods (HbA1c, FBC, liver and renal function) and familiarisation with the testing procedures. On experimental visits, the participants will arrive at 0800 in a fasted state (12 h), and will have refrained from exercise 48 h before, alcohol and caffeine 24 h before, and taking any diabetes medication the morning of the visit. Participants will then undergo: baseline measurements of bloods, central haemodynamics, RMR, macro- and microvascular function; then an oral glucose tolerance test (OGTT) and re-measurements of blood (for analysis of plasma [glucose] and [insulin] only here). After the OGTT participants will be provided with a snack.

The intervention visits consist of the participant being immersed into a 40 °C hot tub for 1 h with rectal temperature measured throughout to ensure target deep body temperature is attained (38.5 - 39°C) and to also monitor deep body temperature for safety (see 10.5). To clamp deep body temperature, body positon in the hot tub will be manipulated accordingly to allow heat loss to occur. If rectal temperature goes above 39 °C the heating protocol will be terminated and cool procedure enacted. Visit measurements and time course are explained in detail in section 10.6.

Participants and Recruitment: A power calculation indicates (looking for a difference in paired means) that 15 people are required to detect a 1 standard deviation change in insulin sensitivity (alpha = 0.05; power = 80%). Given the magnitude of change shown in other chronic passive heating studies [60] it is not unreasonable to expect to see this effect size (see 10.1). To account for participant drop-out we intend to recruit 20 people aged > 35 years with T2DM (as defined by the World Health Organisation (WHO), with an HbA1c > 48 (mmol/mol). Participants will be excluded from the study if they have uncontrolled hypertension (systolic blood pressure > 180 mmHg); a history of myocardial infarction or cerebrovascular events; leg ulcers; unable to give informed consent or any other serious medical condition which would interfere with safety or data interpretation.

Participants will be recruited from a database of known individuals with T2DM who attend outpatient clinics at the Queen Alexandra Hospital (Portsmouth) and via posters and word of mouth within the University of Portsmouth. We will also recruit from local GP practices via mail outs.

Consent and Screening: Visit 1 will act as a screening, consent and familiarisation (~ 1 h). Participants will be given the opportunity to ask any questions they may have after reading the participant information sheet. A standard medical history and clinical examination will be undertaken by a member of UoP staff, including for example height, mass, electrocardiogram, ankle-brachial pressure index and blood sample collection for lipids and biochemical markers, and liver function tests. Seated resting blood pressure measurements will be performed to calculate systolic, diastolic blood pressure.

Will you obtain informed consent from or on behalf of research participants?

A member of the UoP research team will take consent for all subjects. These individuals are familiar with the principles underpinning informed consent and methods to assess capacity to give consent, for example asking the participant to summarise what the study involves for them. Only participants who have capacity to consent to taking part in research will be recruited into the study. If the researchers are in any doubt they will not be consented. The research staff will endeavour to keep the participants informed throughout the study and will encourage participants to ask questions about any uncertainties throughout their involvement in the study.

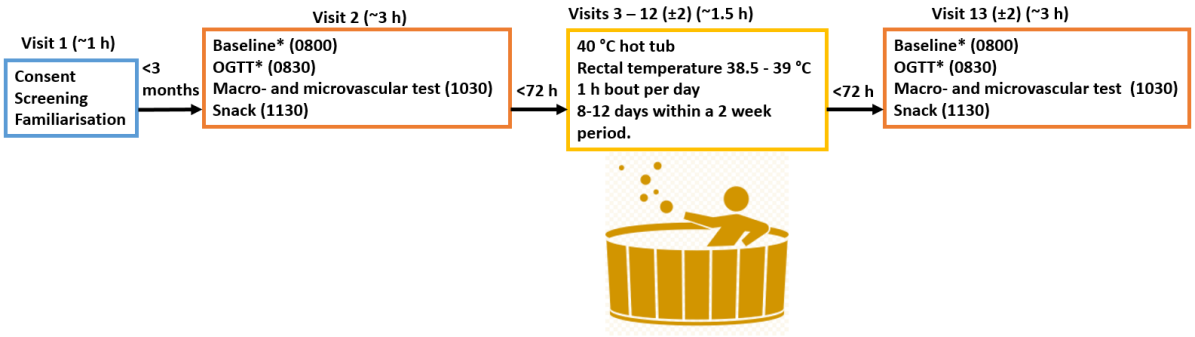

Figure 1. Study overview. OGTT = oral glucose tolerance test. *measurements at baseline will be blood (biomaker analysis detailed in 10.6), central haemodynamics and resting metabiolic rate. At the end of OGTT only blood will be taken again for the measurment for the analysis of plasma [glucose] and [insulin].

7.2 Primary and Secondary Endpoints/Outcome Measures

The primary outcome of this study is insulin sensitivity. Secondary outcomes are:

1) fasting plasma [glucose]
2) AUC plasma [glucose]
3) peak plasma [glucose]
4) $HbA1_c$
5) plasma [eHSP70]
6) plasma [IL-6]
7) plasma [IL-10]
8) plasma [butyrate]
9) plasma [growth hormone]
10) RMR
11) fat oxidation
12) CHO oxidation
13) macrovascular function
14) microvascular function
15) resting heart rate
16) stroke volume
17) total peripheral resistance
18) cardiac output
19) heart rate variability

8. STUDY PARTICIPANTS

8.1 Study Setting

Firstly, the Clinical Exercise Physiology Laboratory at the University of Portsmouth has a successful history of conducting studies in clinical population. 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. Dr Anthony Shepherd is the lead applicant and has expertise in trial management and clinical exercise physiology and his work has focused on exercise, (oxygen uptake, quality of life, functional capacity and glucose homeostasis) in clinical cohorts. Mr Thomas James is a PhD candidate currently working with a background in clinical, exercise and environmental physiology and will be leading most of the data collection and analysis. Mr James has recently completed a similar study in individuals with T2DM. Dr Jo Corbett is highly experienced in running exercise and thermal physiology trials and is a World leader in the area, running 14 studies over 14 years with 74 publications. Dr Corbett will be helping with data interpretation. Dr Maria Perissiou is an early career researcher with extensive experience in assessing the acute cardiovascular responses to exercise in clinical population. Dr Perissiou has expertise in exercise testing, assessments of cardiovascular function and venous cannulation in clinical populations, and will be helping with data collection and interpretation. Dr Clare Eglin is an experienced thermal physiologist and has experience leading MoD trials. Prof. Michael Tipton's World leading expertise in thermal physiology will be used to help with data interpretation. Dr John S. Young's expertise in biochemistry will be used for data analysis and interpretation. Dr Zoe Saynor has expertise in exercise testing and training in clinical populations, venepuncture and cannulation and will help with data interpretation. Prof Michael Cummings (clinical lead for the local NIHR diabetes research network), will be involved in this project and will provide clinical advice, responsible for participant safety and helping with participant recruitment. Ms Sharon Allard and Dr Kathryn Carey-Jones will aid with participant recruitment through Clinics and primary care. Final report and project related publications will be a collaborative effort of the team.

8.2 Overall Description of Study Participants

Participants will be adults with a clinical diagnosis of T2DM, as defined by the WHO. A standard medical history and clinical examination will be undertaken by a member of UoP staff. Information taken during the examination will be height, weight, full blood count (analysed from a blood sample that will be collected), HbA1c and a resting ECG. ECG will be examined for irregularities and a clinical decision will be made on further participation to the study by a consultant at QA hospital.

9. SAMPLING

An *a priori* sample size calculation was performed based on a passive heating study assessing insulin sensitivity in health individuals [60]. The mean difference between pre and post chronic passive heating was 16 ± 14.5 a.u. for insulin sensitivity (~1SD). For 80% power and an α-

level set at $P = 0.05$ (two tailed), to detect a 1 SD difference 15 individuals are required. In order to account for dropout, we anticipated that 20 individuals would need to begin the study.

10. STUDY PROCEDURES

10.1 Recruitment

Our typical recruitment rates via GP practices is approximately one in ten people with T2DM. 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 = 15) people to account for drop outs.

Recruitment will be carried out by members of the UoP research team (or clinical team if participant clinical records are needed) acting with appropriate contracts with the responsible care organisation. The clinical staff will be briefed in detail regarding the current project and they will be provided with the inclusion and exclusion criteria of the study in order to perform any database searches. In the case of the computerised search of GP databases, this will be conducted by the direct healthcare team. Following receipt of the reply slips to the UoP research team, consent and screening will take place and results forwarded (for those willing) to our consultants who will review the results against the inclusion / exclusion criteria. Posters and word of mouth will also be utilised. Screening blood will be analysed at QA pathology or at a commercial pathology laboratory.

Inclusion criteria:

- Male or female (post-menopausal) aged 35 years or above.
- Diagnosed with T2DM as defined by the WHO (≥48 mmol/mol).
- Participant is willing and able to give informed consent for participation in the study.
- Participant is able to understand and fully cooperate with the study protocol.

Exclusion criteria:

- Severe peripheral neuropathy (to the point to which they cannot sense temperature)
- Uncontrolled hypertension (≥180 systolic / 100 diastolic mmHg)
- Taking any medication which may interfere with data interpretation or safety
- Who have had a myocardial infarction or cerebro−vascular event
- Any cardiac abnormalities which restrict hard exercise

- Current smokers or who have stopped within 3 months
- Participant is unable to understand and/or fully cooperate with the study protocol
- Any other serious medical condition which would interfere with data interpretation or safety will be excluded from participation.
- Skin ulcerations
- Eczema
- Pre-existing postural hypertension
- Existing cardiac diseases (identified during screening)

10.2 Participants approach strategy

The clinical team will provide interested parties with an invitation letter and the PIS when they attend the clinic. Additionally, the clinical team may send out an invitation letter including a return slip to potential participants on their clinic list or GP database, who could then opt in to finding out about the study. 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.

10.3 Screening and Enrolment

Consent and Screening: Visit 1 will act as a screening, consent and baseline assessment (~1 h). Participants will be given the opportunity to ask any questions they may have after reading the PIS. A standard medical history and clinical examination will be undertaken by a member of UoP staff, including height, mass, electrocardiogram, full blood count and HbA1c. Seated resting blood pressure measurements will be performed to calculate systolic, diastolic blood pressure.

10.4 Randomisation

Due to study design, randomisation will not be necessary.

10.5 Study intervention

Daily passive heating: Participants will attend the laboratory on 8-12 occasions within a period of 14 days (~ 90 min per visit) with the aim of completing 10, at any point in the day. Anyone completing less than 8 will be excluded from analysis. During each visit participants will self-insert a rectal thermistor and will then be immersed into a hot tub (~ 40 ^{o}C) for 60 min. Body position will be manipulated as required to achieve and maintain a target T_{rec} at 38.5 ^{o}C using 40 ^{o}C water. Aural temperature will also be measured every 15 min for determination of

validity and time lag compared to T_{rec}, for possible use in future studies. BP will also be measured at 15 min intervals after passive heating in the first intervention visit to firstly, establish safety data on the protocol (i.e. to avoid syncope) and secondly, be used as a benchmark for recovery time in subsequent intervention visits. Participants will be required to wear a bathing suit and a t-shirt for all heating visits.

10.6 Study Assessments

Study assessments of the outcome measures will take place before (< 72 h) and after (< 72 h) the intervention. The pre- and post- experimental visits should take ~ 3 hours. Briefly, in, line with OGTT guidelines, participants will arrive at ~0800 following an overnight fast. The research coordinator will confirm with the participants that they have completed a 48 h food and physical activity log, that no exercise has been performed and that no alcohol or caffeine has been consumed on the day before. To ensure that participants arrive in a euhydrated state, they will be asked to consume ≥17 $mL \cdot kg^{-1}$ of water before each visit. Participants will rest for 15 min in a semi-recumbent position, in comfortable ambient conditions (23 °C, 50% relative humidity), then have baseline bloods (collected via venipuncture), ECG, central haemodynamics, RMR collected / measured and brachial artery FMD measurements will start followed by iontophoresis. At ~0830 a 2 h OGTT will start with the ingestion of a glucose drink (Rapilose, 75 g glucose). At the end of the OGTT after bloods have been taken again, participants will be provided with a snack. A detailed description of the outcome measures are given below.

Primary Outcome:

Insulin sensitivity: Venous blood will be drawn in a fasted state after a resting period of 15 min for analysis of plasma [glucose] (Fluoride/Oxalate tubes, BD, USA) and [insulin] (EDTA tubes, BD, USA). An OGTT (75 g glucose solution) (Rapilose OGTT solution, Penlan healthcare, Japan) will then start and after 120 min of rest, venous blood will be drawn again. Insulin sensitivity will then be calculated via the Gutt method [61].

Secondary Outcomes:

Biomarkers

Blood samples will be taken at screening in EDTA tubes for assessment of full blood count and $HbA1_c$. Fasting and area under the curve (AUC) plasma [glucose] will be assessed as a marker of glucose control. Change in circulating plasma [Growth Hormone (GH)] (EDTA, K2, BD, NJ, USA) will be assessed due to its diabetogenic effects via gluconeogenesis and glycogenolysis from the liver and kidneys and suppression of glucose uptake in adipose tissue [59, 60]. Change in circulating plasma [Butyrate] (EDTA K2, BD, NJ, USA) caused from alterations in gut microbiome [64] will be assessed due to its effect on insulin secretion [65],

insulin action [65] and inflammatory status [66]. Change in circulating plasma [eHSP70] (EDTA K2, BD, NJ, USA) will be assessed due to eHSP's role in reducing inflammatory status. Change in circulating plasma [IL-6] (EDTA K2, BD, NJ, USA) and [IL-10] (LH, BD, NJ, USA) will be assessed to determine inflammatory status. Blood samples for assessment of fasting plasma [glucose], $HbA1_c$, [GH], [Butyrate], [eHSP70], [IL-6] and [IL-10] will be from the venous collection at a fasted state and at the end of the 120 min OGTT for AUC plasma [glucose] and insulin sensitivity only. With exception to screening samples which will be taken for analysis immediately after visit, all samples will be immediately placed in a chilled (4°C) centrifuge and spun at 4000g for 10 min. Once spun, the plasma will be pipetted into Eppendorf's and placed into a -80°C freezer for storage. Additional biomarkers may be looked at in future if extra funds become available.

Metabolic measures

Fuel utilisation: Breath-by-breath changes in pulmonary gas exchange and ventilation will be non-invasively measured at rest to enable the estimation of resting metabolic rate (RMR). The assessment of pulmonary gas exchange (e.g. $\dot{V}o_2$ and $\dot{V}co_2$) will give us the respiratory exchange ratio (RER). RER is a reliable and valid measure of fuel utilisation. To assess RMR, participants will be asked to lie down and rest for 15 mins, following this, pulmonary gas exchange will be measured via a gas analysis system for 5 min. Participants will be required to use a mask covering their nose and mouth (no restriction to breathing). In order to assess inspired and expired gas (volume and concentration), gases will be sampled through a capillary line attached to the turbine inside the face mask. A sub analysis of this breath by breath data will give us the respiratory exchange ratio (a value of 0.7 indicates only fatty acid metabolism and values of > 1 indicates only glucose metabolism, any value in the middle equates to a combination of both). This is an accurate assessment of rate of energy expenditure and fuel utilisation, and will be indicative of what proportion of carbohydrate and fat is being metabolised [67]. In order to calculate the RMR and rate of carbohydrate, and fat metabolism the ISO 8996 equations will be used [68]. Pulmonary gas analysis will be performed on all testing visits. Calibration will follow the guidelines given in the user manual produced by COSMED Omnia. Briefly, this involves calibrating the gas analyser by passing through a sample of gas with a known concentration and calibrating the volume on the gas flowmeter by using a 3 L syringe to pass a known volume of air through the flowmeter.

Cardiovascular measures

Macrovascular function: Brachial artery FMD will be used as a measure of endothelial function [69]. Endothelial-dependent function is assessed by measuring FMD in response to a 5 minute ischaemic stimulus, induced by forearm cuff inflation [69]. Measurements will be performed in the supine position on the right arm with the cuff placed distal to the olecranon process. High-resolution duplex ultrasound (SonoSite Edge II, Bothell, Washington, USA) with a 12-MHz multifrequency linear array probe will be used to image the brachial artery at the distal

third of the upper arm and simultaneously record the longitudinal B-mode image and Doppler blood velocity trace. The angle of Doppler insonation will be 60°. Images will be optimized, and settings (depth, focus position, and gain) will be maintained between FMD assessments within each individual visit, and the location of the transducer will be recorded and marked on the skin using an indelible marker. Following a 60-s baseline recording period, the cuff will be rapidly inflated to 220 mmHg and maintained for 5 min (moorVMS-PRES, Axminster, UK). Ultrasound recordings will resume 30 s before rapid cuff deflation (<2 s) and be continued for 3 min thereafter in accordance with recommendations [66, 67]. All ultrasound scans will be performed by a member of the UoP research team (same researcher within participant). Analysis of brachial artery diameter will be performed using custom designed edge-detection and wall-tracking software, which is largely independent of investigator bias. Recent papers describe the analysis approach in detail [66, 67]. Briefly, from recordings of the synchronised artery diameter and blood velocity data, blood flow (the product of lumen cross- sectional area and Doppler velocity) will be calculated at 30 Hz. Shear rate (an estimate of shear stress independent of viscosity) will be calculated as four times the mean blood velocity/vessel diameter via commercially available software.

Microvascular function:

Individuals will be acclimated for a minimum of 30 minutes in an ambient temperature of 23 ºC prior to acetylcholine (ACh) and insulin being delivered transdermally via iontophoresis to volar aspect of the left forearm as previously described [71].

Briefly, following cleaning of the skin surface with water for injection, two perspex rings will be attached to the skin with one acting as an anode, and the other as the cathode. These electrodes will be connected to the iontophoresis controller (MIC 2, Moor Instruments, UK). Both chambers have an 8 mm inner diameter. The anode chamber will be filled with ~ 0.5 mL of ACh (Braun, Melsungen, Germany), with a 1% concentration dissolved in water for injection. The cathode chamber will be filled with ~ 0.5 mL of insulin (Sigma-aldirch, Missouri, USA) with a 0.01% concentration dissolved in water for injection. The protocol for electrical pulses included: four at 25 µA, followed by a single pulse of 50 µA, 100 µA, 150 µA and 200 µA. These pulses will last for 20 s with 120 s intervals between each pulse where no current will be applied. An interval of five minutes will be given between testing each site (forearm, finger and foot).

Laser doppler probes (VP1T / 7, Moor Instruments, UK), connected to a perfusion monitor (moor VMS-LDF, Moor Instruments, UK) will be used to assess skin blood flow. Data will be recorded using an acquisition system (Powerlab, AD Instruments, Australia) and software (LabChart 7, AD Instruments, Australia). The laser doppler probes will be secured in the Perspex rings prior to the iontophoresis protocol on the forearm and on the corresponding site on the contra-lateral limb (to differentiate between local and systemic responses). Skin blood

flow responses will be expressed as CVC (CVC = skin flux/MAP; flux·mmHg^{-1}). The average skin blood flow for both ACh and insulin will be calculated over the final 20 s of the intervals between each successful pulse (i.e. 100-120 s post each pulse) [71]. Maximal skin blood flow, taken at the highest point which will not always be following the final pulse and area under the curve (AUC) will be calculated for each participant. Skin temperature (T_{sk}) will be recorded with skin thermistors (Grants Instruments, Cambridge) placed next to the Perspex chambers. BP will be measured on the contra-lateral arm to the site of iontophoresis using an automated BP monitor (Omron M5, Omron, Milton Keynes, UK) before and after each iontophoresis protocol to calculate mean arterial pressure (MAP).

ECG: HR will be automatically calculated from the ECG (Dynascope, Fukuda Denshi, Japan). Heart rate variability (HRV) will be calculated as a low frequency (LF) variable and root mean square of successive differences (RMSSD). LF has been chosen as it indicates a physiological origin of both sympathetic and vagal activity. RMSSD is then being calculated additionally as it gives a stronger representation of vagal tone (due to it being affected much less by respiratory sinus arrhythmia (RSA)) than LF thus improving validity of the vagal tone measure [72]. Both measures will be calculated from an ECG trace (Kubios HRV software, Kubios, Finland).

Other measures

Central haemodynamics: Using thoracic bio-reactance technology (Physioflow), which will derive heart rate, stroke volume, cardiac output and total peripheral resistance, at rest. Four electrodes are placed on the participant's chest between which a small, painless current is passed.

10.7 Discontinuation/Withdrawal of Participants from Study Treatment

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

10.8 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

Daily passive heating: Participants will attend the laboratory on 8-12 occasions within a period of 14 days (~ 90 min per visit) with the aim of completing 10, at any point in the day. Anyone completing less than 8 will be excluded from analysis. During each visit participants will self-insert a rectal thermistor and will then be immersed into a hot tub (~ 40 ^{o}C) for 60 min. Body position will be manipulated as required to achieve and maintain a target T_{rec} at 38.5 ^{o}C using 40 ^{o}C water. Participants will be required to wear a bathing suit and a t-shirt for all heating visits.

11.2 Adherence to Study Treatment

The research coordinator will confirm that the participants have completed at least 8, 1 h passive heating sessions between experimental visits.

11.3 Accountability of the Study Treatment

Due to the nature of the study, blinding of the participants will be impossible.

11.4 Concomitant Medication / Therapies

Medication will be recorded on enrolment and updated throughout their time on the trial. For each experimental visit, hypoglycaemic aids will be stopped for the morning (see pre experimental instructions for details).

12. ASSESSMENT OF SAFETY (IF APPLICABLE)

12.1 Definitions

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 screening, consent and baseline assessment (~1 h). All measures for this visit will be stored in the clinical records 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 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 Anthony Shepherd) is responsible for database maintenance and management.

13.3 Record keeping

Participant consent forms will be kept for 30 years and then destroyed in accordance with University of Portsmouth guidelines. Data and documentation will be kept for 10 years and then reviewed. Data storage allows us to undertake any necessary or retrospective data analysis. It

also allows us to keep records for people who have indicated that they will be willing to participate in studies in the future. We will destroy any details if requested. The data will be stored in the School for Sport and Exercise Science at the University of Portsmouth. The unit is secured with key codes and locks and is alarmed out of hours. All computer files are password protected and only study investigators will have access to these data.

14. DATA ANALYSIS

14.1 Description of Analysis Populations

Participants who complete both experimental visits and at least 8 intervention visits will be entered into the analysis.

14.2 Analysis of Endpoints

All data will be tested for normality. Where data are not normally distributed, a nonparametric test will be performed. Data will be presented as means ± SD. Statistical analyses will be performed on SPSS software version 24.0 (Chicago, IL). Statistical difference will be accepted when $P < 0.05$. Statistical differences will be assessed by paired samples t-test or Wilcoxon signed –rank test (for non-parametric data).

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 study a statistical analysis plan is not required (excluding the above).

14.5 Interim analysis and criteria for early study termination

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

15. ETHICS

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 participant identification centre forms will be forwarded to the R&D department at Queen Alexandra Hospital Portsmouth, prior to recruitment of participants. Written informed consent will be obtained from all participants. Insurance indemnity will be provided by the University of Portsmouth for this study.

Every effort has been made to keep the risks and discomforts to a minimum, with first aid cover and extensive screening procedures in place 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.

The main burden to participants is a 12 h fasting period before their experimental visits. Participants will be asked to cease all hypoglycaemic agents on the morning of testing and will be instructed to bring them with them on the day. This is in line with a normal fasted OGTT typically used in clinics. Prior to leaving the laboratory, participants will be provided with lunch, they can take their tablets and blood sugars checked prior to leaving.

We will also be asking people with T2DM to be immersed in 40 °C water for all intervention visits. This may cause some discomfort which will be assessed with a thermal sensation and comfort scale. We will be measuring the core temperature via rectal thermistor and monitoring it closely at all times. Participants will be reminded that they are free to withdraw at any time point and will also be withdrawn and cooled if T_{rec} raises above 39°C (as stated in the school SAP's).

Participants will be asked to insert a rectal thermistor on 8-12 occasions (water immersions). This can be uncomfortable upon insertion; however, this is necessary for safety purposes. Participants will be provided with lubrication and instructions.

Other less burdensome tests will be performed such as:

Blood pressure; participants will feel a cuff squeeze their arm and this can be uncomfortable for a few moments. Blood samples taken via intravenous access can cause discomfort associated with insertion of the needle.

Indelible ink: Participant will be asked to keep remarking their arm with indelible ink where the ultrasound probe was used, for just over 2 weeks. This could cause slight annoyance over the 2 week duration.

Other risks that have been accounted for are hypotension and loss of consciousness in the water. If at any point the participants BP is measured at ≥ 180/100 mmHg the trial will be terminated and the participant withdrawn from the study. If there is loss of consciousness in the water the participant will immediately be removed securely via (SAP's). This can be done quickly as they will already be lying on a sling. Once they are out of the water and placed on a bed, their breathing will be checked and first aid administered. They will also be cooled via cold water spray and a fan. Additionally, their head will never go underwater as their head will be elevated above waterline throughout all immersions.

At consent participants will be asked "I agree to having my photo taken for the purposes of publication and also presentation at conferences (all identifying features will be blacked out i.e. your face)". Participants have the option to agree or decline. This is to mainly aid in the presentation of the methodology in papers and conferences if necessary.

In the event of any adverse event, researchers follow IMO approved SOP's. The process in brief, involves researchers administering first aid and while another then goes and dials "3333" on the laboratory phone. This number directly links to the ambulance service who will automatically know where to come (~10 min arrival time). The other researcher will then go and seek help from another first aid member of staff in the building. The participant will have first aid administered until paramedics arrive.

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

Refer to declaration and that the study protocol will be carried out in accordance with it.

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):

Our team has a strong track record of patient, carer and public involvement (PCPI) work. Dr Shepherd has been involved in a PCPI award winning project presented at the UK Stroke Forum (2016). At least 15 individuals with T2DM have been consulted (during a local conference for World Diabetes Day and DESMOND) and have stated that they liked the idea of that going in a hot tub could improve their health. Some concerns over travel funds were

raised and over the number of visits in the week (for those that work). In response we have made weekend testing available, we will offer travel reimbursement to be as inclusive as possible. The results of this project will be disseminated through publication in peer reviewed scientific and clinical journals and via presentations at local, national and international meetings. A summary of the results will be sent to all the participants.

16.2 Study implementation

Issues will be managed by the senior members of the UoP research team.

16.3 Dissemination

We endeavour to disseminate findings through local groups, local/national media, research papers and international conferences.

17. FINANCING AND INSURANCE

17.1 Research Costs

The majority of consumables and personnel required for testing have been funded by the University of Portsmouth. All hardware and software is already available.

17.2 Service Support Costs

No service support costs are being sought.

17.3 Excess Treatment Costs

No excess treatment cost are being sought.

17.4 Study Sponsorship

This study is being sponsored by the University of Portsmouth.

18. TIMETABLE AND ORGANISATIONAL CHART

November 2020: Funding for consumables has been secured
November 2020: Application for ethical approval
February 2021 - October 2021: Participant recruitment
March 2021 – February 2022: Data collection
February 2021 – April 2022: Data analysis
April 2021: Study completion date
Post-April 2022: Dissemination of results through conference proceedings, journal submissions and practitioner outlet

19. RESOURCES, EQUIPMENT AND PHYSICAL FACILITIES

In addition to the school support, and in order to facilitate the completion of the work, the UoP research team will have full access to all of the necessary world-leading extreme environment facilities that are housed within the school estate given that they are pivotal to the success of the project. Specifically, this will enable temperature control within the facilities to ensure reproducibility. Finally, the school will ensure that full technical assistance is provided to enable the set-up of the laboratories and testing rooms.

20. DISSEMINATION AND OUTCOME

The research is to be disseminated in internationally recognised, peer reviewed journals and at relevant scientific conferences, as well as contributing to Mr. Thomas James' PhD thesis. Furthermore, all screening data will be utilised as part of the participants's annual report, and distributed to clinicians and participants upon request.

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