**DEAKIN UNIVERSITY HUMAN RESEARCH ETHICS COMMITTEE**
**PROJECT DESCRIPTION/PROTOCOL**

**Instructions for preparing the project description/protocol**

1. The purpose of the Project Description is to provide the scientific and academic background and context of a research project.
2. A Project Description is a **mandatory** component of a submission using the Human Research Ethics Application (HREA).
3. The section headings in this Project Description template represent a structure for presentation of information about a research project that meets the needs of an ethics review body.
4. Not all headings or sub-headings in this template are relevant for each research project. Where a question is not relevant please enter NA into the response box. Please do not delete the question.
5. Researchers may use visual aids embedded in the project description/protocol to assist in describing their project where appropriate (e.g. images, videos etc.).
6. Submissions of clinical trial proposals may use alternative protocol templates, such as the SPIRIT statement.
7. Researchers may choose to submit an existing document (such as a protocol or project description that has already been developed) instead of developing a new document.
8. If researchers choose to submit an existing document instead of using one of the templates provided, they may need to provide indications to the ethics review body of where in the submitted document the content corresponding to the relevant fields in the template are located.
9. There is no need to duplicate information in the HREA into the Project Description or vice versa.
10. Language that is understandable to non-technical reviewers should be used.

## COVID-19

All research must comply with current COVID-19 restrictions, as well as with Deakin's COVIDSafe Management Plan. Any activities considered as having high COVID-19 risk (e.g. requiring safety measures over and above the COVIDSafe Management Plan and risks covered by the general requirements of entry to campus) must have an approved COVIDSafe Activity Plan in place. This includes any on-campus research involving a face-to-face element, as well as off-site research (e.g., site visits, fieldwork etc).

Please indicate if your project complies with:

☒ The current COVID-19 restrictions,

☒ Deakin's COVIDSafe Management Plan and

☒ Any applicable COVIDSafe Activity Plan in place for high COVID risk activities.

☐ N/A - the restrictions are not applicable due to the nature of your project (e.g., online survey).

☐ None of the above – please provide some additional information. Click or tap here to enter text.

☒ Where your project may include COVID-19 related risks, please acknowledge that you have taken into consideration the information provided on the FAQs - Human Research Ethics site.

Will you include direct or indirect questions related to the participants' lived experience of COVID-19? ☐Yes ☒No

If yes, tick below to confirm you have:

☐ Included an appropriately tailored version of the following statement in your Plain Language Statement:

**Low risk projects only**

*"While it is not expected that participating in the research will cause you to feel distress, we recognise the challenging circumstances the COVID-19 pandemic has caused for many community members. As such, we would like to highlight that if you, or those close to you are experiencing distress, or are in need of additional support, you are encouraged to contact [insert appropriate contact details for your participants e.g., Beyond Blue, Lifeline, Suicide Call Back Service, Headspace, Kids Helpline etc]."*

**Higher than low risk projects**

*"In addition to the risks outlined in this document, we recognise the challenging circumstances the COVID-19 pandemic has caused for many community members. As such, we would like to highlight that if you, or those close to you are experiencing distress, or are in need of additional support, you are encouraged to contact [insert appropriate contact details for your participants e.g., Beyond Blue, Lifeline, Suicide Call Back Service, Headspace, Kids Helpline etc]."*

**For all projects including direct or indirect questions related to the participants' lived experience of COVID-19, please check boxes below to confirm:**

☐ You have included the same statement (above) at the conclusion of your survey/ questionnaire/ research instruments.

☐ You will immediately review all research data for any disclosures of heightened distress, suicidal ideation or attempts or self-harm. Researchers who are unable to review their data immediately or who need clarification on what kind of time frame constitutes an acceptably prompt review, should contact research-ethics@deakin.edu.au to discuss their options.

☐ You will report disclosures of heightened distress, suicidal ideation or attempts or self-harm as per the FAQ website, to research-ethics@deakin.edu.au.

# 1. Project details

**Title:** The effects of antioxidants on vascular function and exercise capacity in peripheral artery disease.

**Acronym:** Ex-PAD

**Protocol version number*:*** 1

**Project overview:** Peripheral artery disease (PAD) can result in extensive functional alterations including vascular dysfunction, reduced capacity to exercise, and reduced quality of life [1]. The progression and cause of PAD is thought to be associated with increased oxidative stress and tissue injury related to increased production of reactive oxygen species (ROS) [2] and a decrease in antioxidant defences [3]. Oxidative stress and inflammation are major features in the development of atherosclerosis and are therefore extremely relevant in the development of PAD [4, 5]. Several important enzymatic antioxidant defences have been shown to be deficient in the muscle of PAD individuals compared to controls [3]. Endogenous antioxidants function as defences to remove or regulate ROS production. Therefore, antioxidant deficiencies can lead to a state of excess oxidative stress which contributes to vascular dysfunction, decreased exercise capacity, and the development of PAD, cardiovascular disease, and cerebrovascular disease [6-9]. However, few studies have adequately explored antioxidant treatment in PAD patients, and none have explored antioxidant treatment and the effects on the extensive vascular network during exercise. The aims of this developmental work are to directly test whether antioxidant treatment (via the intravenous infusion of an antioxidant) can acutely improve vascular function and exercise capacity in patients with PAD.

# 2. Project team roles & responsibilities

**Chief Investigator:**

1. **Dr Hannah Thomas (Deakin University)** is a Research Fellow in the School of Exercise and Nutrition Sciences at the Institute for Physical Activity and Nutrition (IPAN) at Deakin University. Dr Thomas will lead the project and be responsible for obtaining study ethical clearance, piloting, participant recruitment, obtaining consent, data collection and analysis, and initial drafting of the primary manuscript. Dr Thomas is an Accredited Exercise Physiologist with 5 years of experience in exercise rehabilitation of cardiometabolic disease patients. Dr Thomas has extensive experience conducting research that involves exercise testing, metabolism, and vascular imaging in a range of populations including healthy individuals and those who suffer from cardiometabolic diseases. She has extensive experience co-ordinating large human clinical trials in exercise physiology and cardiovascular physiology. Dr Thomas has completed training in all the techniques required for successful and safe completion of this study.

**Associate Investigators:**

1. **Dr Lewan Parker (Deakin University)** Dr Parker has considerable experience conducting invasive studies in healthy and clinical populations and accrued extensive exercise physiology, biochemical, and data analytical skills. Dr Parker has current CPR and First Aid training, is qualified to perform cannulation and venepuncture, and has experience with a range of exercise physiology related research equipment. Dr Parker has completed training in all the techniques required for successful and safe completion of this study. Dr Parker has assisted with study design and will assist with data collection, analysis, and interpretation, and dissemination of the overall project findings.
2. **A/Prof. Michelle Keske (Deakin University)** is internationally recognised for her significant contribution in the field of muscle microvascular blood flow in response to exercise and insulin. Her research strongly implicates microvascular dysfunction in muscle in the development of insulin resistance, type 2 diabetes and exercise intolerance. She is highly

experienced with the contrast-enhanced ultrasound (CEU) technique and has previously conducted invasive exercise training clinical trials in cardiometabolic patients. A/Prof Keske has assisted with study design and will assist with data collection, analysis, and interpretation, and dissemination of the overall project findings.

3. **Dr Andrew Garnham (Deakin University)** is a medical doctor with extensive experience in physiology, sports medicine, and nutrition. Dr Andrew Garnham has previously provided his medical expertise and knowledge on previous studies conducted at Deakin University, including antioxidant treatment and intravenous infusions of the ultrasound Contrast Agent (DUHREC# 2019-202, 2021-048, and 2019-426). Dr Garnham has assisted with study design and the safety evaluation of the study and will assist with participant support and counselling in the event of any adverse events or side effects experienced by the participants.

## 3. Resources

This project is funded by the Institute for Physical Activity and Nutrition (IPAN) seed funding scheme at Deakin University (funding commenced February 2022). The equipment required for this project is already currently available (or has been ordered) at Deakin University.

## 4. Background

In addition to the known detrimental effects on the lower extremities, PAD is often associated with atherosclerotic disease in other vascular beds, including coronary and cerebral arteries [10, 11]. PAD causes a 60% excess risk of all-cause mortality, 96% increase in cardiovascular deaths, 45% increase in the risk of developing coronary artery disease, and 35% increase in the risk of developing a cerebrovascular disease [12]. Despite this, minimal research outside of the large arteries in the lower limbs has been done. To treat vascular health and function in PAD patients more effectively, the vasculature should be thought of as an integrated and dynamic system (inclusive of the cerebral arteries and the microvasculature). This study will be the first to investigate extensive vascular function and health in PAD patients (brain, heart, large peripheral arteries and smallest vessels in the skeletal muscle). Furthermore, oxidative stress is reported to play a major role in PAD development and disease progression [2, 3]. In some cases, antioxidant treatment is effective at improving large artery function [4]. However, research-to-date has yet to investigate the effects of antioxidants on the extensive vascular network which we now know is equally important for exercise capacity and cardiometabolic health [13, 14]. We plan to use a novel approach (intravenous infusion of an endogenous antioxidant) to acutely decrease oxidative stress in PAD patients at rest, and while they undergo a maximal exercise test. Combined with modern techniques in vascular imaging, this study will be the first to determine the effects of antioxidant treatment on exercise capacity and vascular function in PAD patients.

**Aim.** This study aims to:

- Test whether antioxidant treatment (via the intravenous infusion of an antioxidant) can improve vascular function and exercise capacity in patients with PAD.

**Hypotheses.** We hypothesise that:

1. Antioxidant treatment will improve exercise capacity in individuals with PAD.
2. Antioxidant treatment will improve vascular function in PAD.

**Expected outcomes.** We aim to show that:

- Infusion of antioxidants during acute exercise can increase blood flow through the body and increase exercise capacity in PAD patients.

We hope findings will provide the foundation for larger future projects including:

- Explore the influence of chronic antioxidant treatment (3 months) on exercise tolerance, vascular function and health and quality of life in PAD.
- Investigating the effects of exercise training (3 months) to determine the impact of reducing oxidative stress on vascular health and function, exercise capacity, and quality of life in PAD patients.

## 5. Project design and methodology

This project will be conducted at the Institute of Physical Activity and Nutrition (IPAN), Deakin University Burwood campus (Building J), in the level 5 exercise laboratory.

***Study overview.***

Participants will be asked to attend a screening and familiarisation session followed by two 2.5-hour testing sessions in a randomised cross-over order, separated by a minimum of 1 week. The testing sessions will require participants to undergo a maximal exercise capacity test on a treadmill with either saline infusion (control) or an antioxidant infusion (antioxidant treatment).

- **Screening and familiarisation session.**
    - Discuss details of the project and what it means to be involved.
    - Obtain informed consent.
    - General health, physical activity, and medical history questionnaires (refer to appendices).
    - Complete ankle brachial index (ABI) assessment.
    - Familiarise participants with the treadmill and ultrasound equipment to be used in the main testing sessions.
- **Visits 2 and 3 – Main testing sessions: Undergo exercise test with and without antioxidant infusion (~2.5 hours each).**
    - Height and weight measurement.
    - Intravenous infusion of glutathione (antioxidant treatment) or saline (non-antioxidant control treatment).
    - Completion of a maximal exercise test on a treadmill.
    - Blood sampling and ultrasound contrast agent infusion via a vein.
    - Blood pressure measurements.
    - Indirect calorimetry (face mask and gas analyser) to measure expired gases.
    - Ultrasound measurement of the heart, brain and thigh (arterial and skeletal muscle microvascular blood flow).

***Participant Recruitment.***

Study participants/volunteers will be recruited via posters (refer to appendix) located in common areas at the Deakin University Burwood campus, local General Practices, the Institute for Physical Activity and Nutrition (IPAN) public website, IPAN public Facebook and Twitter pages and the surrounding Eastern suburbs of Melbourne. There will be no targeted recruitment of students or staff at Deakin University. Volunteers responding to online or print media will be invited to contact Dr Hannah Thomas by telephone or email. Initial screening will be based on basic health and medical history questions by telephone and/or email to rule out major exclusion criteria. Participants who meet the initial inclusion/exclusion criteria will be provided with the Plain English Statement/Consent Form via email or mail and invited to take part in the study. A research investigator will then discuss

the information and consent form in detail (via phone, email or in person) and provide the participant the opportunity to ask any questions they may have. Volunteers will then be given as much time as necessary to consider their participation and be advised to discuss it with their family and friends. If volunteers still wish to participate, then a research investigator will obtain informed written consent (refer to appendix) and the participants first visit will be scheduled.

***Participant Consent.***
Only named investigators on the approved ethics application will be involved in obtaining informed written consent. No information or data will be collected before consent except for questions on major exclusion criteria which will be asked over the phone or email (e.g., history of heart attack or stroke, age, weight estimate etc.). All people will be treated with the same level of respect and dignity. Potential participants will be informed that involvement is entirely voluntary and that if they choose not to partake in the study, this will not disadvantage them in any way. Participants will be excluded if they cannot understand their involvement in the project (including risks and benefits) or provide their own consent. For individuals whose primary language is other than English, if there is a question that there could be any misunderstanding due to inadequate English, participants will not be included.

***Inclusion Criteria:*** Aged 40-75 y, BMI ≤35 kg/$m^2$, ankle brachial index (ABI) of ≤0.90, history of stable intermittent claudication >1 year.

***Exclusion Criteria:*** History of myocardial infarction, stroke, dementia, or respiratory disease, non-cardiovascular barriers to exercise, critical limb ischemia or foot ulcers, uncontrolled hypertension, identification of any medical condition requiring immediate therapeutic intervention and/or current or previous smoker (within the last 12 months).

***Power Analysis:*** The proposed project is experimental by design. No one has performed intravenous infusion of the antioxidant glutathione in PAD patients during maximal exercise. We have previously been able to detect significant alterations in microvascular blood flow in muscle (post-exercise and even post-meal ingestion where blood flow only increases minimally) in healthy adults with samples sizes of 8 and 10 [15, 16]. As such, our method to measure microvascular blood flow is very sensitive. Due to the nature of the project and robust techniques involved (intravenous infusion in a clinical population and modern vascular imaging techniques) we expect to be able to detect a worthwhile and meaningful change of 10% in microvascular blood flow post-exercise with 10 participants. Importantly, the collection of pilot data from 10 participants would be used to directly inform power calculations for future external funding applications – a critical outcome and aim of the seed funded project.

## 6. Project protocol

A detailed description of specific procedures used in the study are provided in a later section called "*data collection and techniques*".

***Screening and familiarisation session (visit 1, ~1 hour).***
Participants who have expressed interest in participating in the study will be provided with the plain language statement/consent form in person or via email, and the study will be explained in detail and an opportunity provided for potential volunteers to ask questions.
Prior to participants signing the plain language statement and informed consent form, the study will again be explained in detail and an opportunity provided for potential participants to ask questions. After obtaining signed informed consent, participant eligibility for study participation will be assessed. Screening will involve:
i) Blood pressure and ABI: Brachial blood pressure and ABI measures will be taken in triplicate and will be recorded with a validated device. Measures will be taken with a correct sized cuff, after ten minutes rest.
ii) Anthropometrics: We will measure body weight and height
iii) Lifestyle: Participants will complete a general health and medical history questionnaire (refer to appendix), and a physical activity questionnaire (refer to appendix).

Following screening assessments, participants will be familiarised with the equipment that will be used in the following two visits (ultrasound machines, treadmill and indirect calorimetry face mask).
The participants next two visits will then be arranged (visits 1 and 2) which they will undergo in a randomised fashion.

***Maximal exercise test with/without antioxidant infusion (visit 2 and 3, ~2.5 hours each).***

After screening for eligibility, participants will attend the laboratory for two study sessions in a randomised cross-over fashion with a minimum one-week washout period between visits. For each testing visit (all 2 visits), participants will be asked to avoid moderate to vigorous-intensity exercise and alcohol for 48 h prior to attending the Deakin University research facility. They will also be asked to avoid caffeine on the day of the visits, and fast for two hours before coming into the research laboratory for each session (i.e., no eating or drinking, except for water, in the two hours leading up to testing). Upon arriving in the exercise laboratory (Building J, Level 5, Deakin University [Burwood]) after a two hour fast, participants will be asked to rest on a hospital bed while an intravenous cannula is inserted into one arm for antioxidant/saline infusion, and in the contralateral arm for blood sampling and infusion of the ultrasound contrast agent. After baseline measurements (e.g., heart rate, and blood pressure) and a resting blood sample is taken, the infusion of the saline or antioxidant solution will commence. After 1 hour of infusion, participants will begin their maximal exercise test on a treadmill, throughout which the saline or antioxidant infusion will continue. Vascular ultrasound measures of the heart and brain (cardiac and cerebral) and venous blood samples will be conducted before infusion begins, before exercise (60 mins after commencing the infusion), immediately post-exercise and 30 minutes post-exercise (Figure 1). An additional blood sample will also be taken at 15 minutes post-exercise. Vascular ultrasound measures of the thigh (microvascular in the skeletal muscle) will be taken immediately before exercise, immediately post-exercise, and 30 minutes post-exercise (three time points only). Indirect calorimetry will be done throughout the exercise bout. Participants will be monitored for 30 minutes after cessation of the infusions to ensure they are feeling fine prior to leaving the laboratory. Importantly, a similar series of infusions has safely been performed in previous studies conducted by the research team ([15, 17]; DUHREC: 2018-010 and 2019-202).

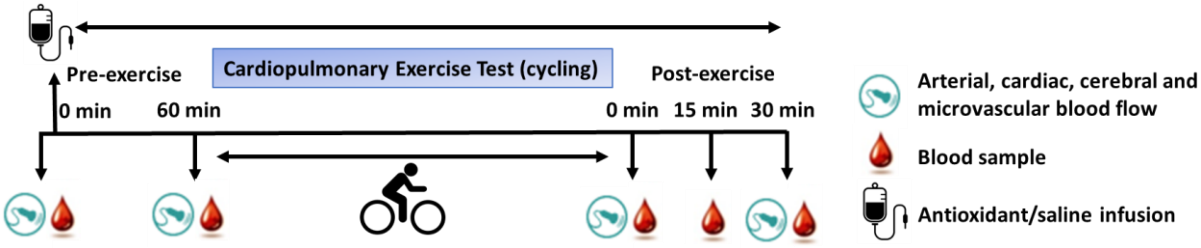



**Figure 1: Protocol for main testing sessions (2 and 3)**

## 7. Data collection techniques

***Cardiopulmonary maximal exercise test.***

Participants will undergo a graded exercise test to exhaustion on a treadmill using standard clinical exercise testing and indirect calorimetry equipment [18, 19]. The test is completed at 3.2 km/h and begins at a grade of 0% for the first 5 minutes and then is increased by 3.5% every 3 minutes until subjects reach their maximum exercise capacity.

***Glutathione antioxidant infusion protocol.***

Glutathione is an antioxidant that is endogenously synthesised in humans and plays a critical role in maintaining cellular redox homeostasis [20, 21]. Several cardioprotective antioxidant defences are deficient in the skeletal muscle of individuals with PAD compared to controls [3]. Both in PAD and in other clinical populations, antioxidant treatment using mitoquinone mesylate, flavonols and vitamin C [4] have led to improved health outcomes including vascular function, leg pain, walking time, carotid

atherosclerosis, and cardiovascular mortality. We will infuse the antioxidant of L-Glutathione (reduced glutathione) at a rate of 15 mg/min over 2.5 hours (approximately 2,250 mg). This infusion rate is similar to what has previously been used in healthy adults and adults with hypertension [20, 22], for much longer durations (e.g., up to 6 hours [20]). Much higher doses have also been safely used in healthy adults and type 2 diabetes patients [21]. Importantly, we have already optimised this dose and infusion rate in healthy adults in our laboratory without any adverse effects (DUHREC: 2019-202 and 2021-048)

***Placebo infusion protocol.***

Saline solution (0.9% NaCl), which is an inert salty solution, will be infused at the same rate as the glutathione infusion.

***Cardiac function and cerebral, thigh arterial and microvascular blood flow assessment.***

**Cardiac function** will be measured via echocardiographic assessment of the heart using a commercial ultrasound machine. Conventional and colour tissue Doppler apical views will be acquired by standard methods as previously performed [23]. Left ventricular systolic and diastolic functional reserve indexes will be derived from changes in systolic and early diastolic colour tissue Doppler velocities. Cardiac index reserve and its constituents (stroke volume and chronotropic indexes) and left ventricular filling pressure (ratio of early diastolic mitral inflow and annular velocities) will be measured.

**Cerebrovascular blood flow.** Intra-cranial blood velocity recordings of the middle and posterior cerebral arteries will be measured using non-invasive transcranial Doppler. Extra-cranial blood flow measures of the internal carotid and vertebral arteries will be collected using a high frequency linear array transducer interfaced to the ultrasound system. Diameter will be assessed using 2D ultrasound and velocity assessed by Doppler ultrasound. Internal carotid and vertebral artery blood flow (ml/min) is calculated as πr2 x mean velocity x 60. Where radius (r) is cm and mean velocity is cm/s. Recordings of middle cerebral artery velocity and posterior cerebral artery velocity will be made at a frequency of 100Hz, at recommended depths, described in TCD guidelines [24]. To standardise vessel recording sites between testing sessions, photographs will be taken of the probe positions, transcranial Doppler settings and velocity traces. Dr Hannah Thomas has extensive experience using this technique [14, 25].

**Femoral arterial** diameter and blood velocity will be measured non-invasively using a high frequency L12-5 linear array transducer interfaced to the ultrasound system as described previously by Dr Parker and A/Prof Keske [15, 17]. Diameter is assessed using 2D ultrasound and velocity assessed by Doppler ultrasound. Femoral artery blood flow (ml/min) is calculated as $\pi r^2$ x mean velocity x 60. Where radius (r) is cm and mean velocity is cm/s.

**Microvascular blood flow** will be measured via contrast enhanced ultrasound imaging during contrast agent infusion, previously performed by Dr Parker and A/Prof. Keske [15, 17] and approved at Deakin University (DUHREC 2017-172, DUHREC #2019-426, DUHREC #2018-010, DUHREC #2021-048, DUHREC #2018-177, DUHREC #2018-382). The technique involves intra-venous infusion of a commercially available contrast agent (Definity, Lantheus Medical Imaging) composed of haemodynamically inert, perflutren lipid microspheres sufficiently small in size to perfuse capillaries/microvasculature. Definity is indicated in diagnostic ultrasound imaging – both liver/kidney assessment (lesion characterisation) and echocardiography (chamber opacification, endocardial border definition and regional wall motion assessment) (Therapeutic Goods Administration approved). A/Prof Keske and Dr Parker have completed ~500 infusions without any adverse events and this technique has been used previously in individuals with PAD without adverse events [26]. A standard ultrasound machine will be used to image the vastus lateralis muscle (thigh muscle group) in cross-section. Depth, gain and focus will be optimised for each participant and maintained during repeated imaging sequences. Standard contrast agent dilutions (1 ml of Definity diluted in 30 mL of saline) and infusion rates will be used (2.0–3.5 mL/min; infusion rate based on body weight) using a standard

syringe pump, as previously done [15, 17]. Images will be acquired using ultrasound and analysed using propriety ultrasound software (QLabs). The acoustic signal generated from the contrast agent microspheres will be measured and is directly proportional to the number of capillaries open/active and volume of blood in the microvascular system. After a high energy pulse of ultrasound, all contrast agent microspheres within the ultrasound beam are destroyed. The rate of contrast agent microspheres reappearance within the ultrasound beam provides an indication of microvascular blood velocity which, combined with microvascular blood volume measurements, is used to determine total microvascular blood flow (i.e., microvascular function).

***Ankle brachial index (ABI).*** Measures of blood pressure and ABI measures will be made in triplicate to screen for PAD. Resting brachial blood pressure will be measured using an automated blood pressure monitor after a period of supine rest. Resting ankle systolic blood pressure will be measured using a handheld Doppler ultrasound probe and sphygmomanometer. The ABI of each leg will be calculated as the higher ankle pressure (dorsalis pedis or posterior tibial artery) divided by the higher brachial pressure (left or right arm) [27]. The ABI is considered an indicator of the severity of PAD based on the level of ischaemia, with a ratio of 1.00 to 1.29 considered normal, a ratio equal to or lower than 0.90 used to confirm diagnosis, and a ratio equal to or below 0.40 often associated with limb-threatening ischaemia [28].

***Blood pressure (brachial and central) and aortic stiffness.*** Brachial blood pressure, central blood pressure and aortic stiffness will be recorded non- invasively with a validated Mobil-O-Graph device (from I.E.M.; http://www.iem.de/en/products/mobil-o-graph.html) as described previously by A/Prof Keske [29]. Measures will be taken with a correct sized cuff. This is an automated device making data collection easy and non-invasive.

***Venous blood sampling.*** Research staff qualified to perform cannulation and venepuncture will collect blood samples via intravenous catheter and venepuncture. Catheters are used when several blood samples are needed from one site over a brief duration such as to be used here. Once the catheter is in place, it is a simple and painless procedure to remove further blood samples. In between each sample, the catheter will be flushed and left filled with a small volume (~1ml) of isotonic saline solution to maintain catheter patency. Catheterisation and blood sampling is a well-established and accepted technique routinely performed at Deakin University.

***Blood analysis.*** Human plasma and red blood cells will be analysed for markers of oxidative stress (e.g., hydrogen peroxide, oxidised glutathione, and F2-isoprostanes) and antioxidant activity (e.g., vitamin C and E, catalase, superoxide dismutase, and glutathione), as previously performed by the research team [30-39]. Venous blood samples will be analysed for markers of vascular function and cardiometabolic health (e.g., markers of inflammation and bone, fat and glucose metabolism), and exercise metabolism (e.g., lactate and oxygen saturation).

***Data analysis.*** Data will be checked for normality and analysed using Graphpad Prism Software. Comparison of multiple means will be analysed using a two-factor repeated measures ANOVA with "Time" (timepoint during the sessions) and "Condition" (Saline [placebo] or antioxidant infusion session) as the within-subject factors. Significant main effects and interactions will be analysed Post Hoc with adjustment for multiple comparisons. Statistical analysis will be conducted at the 95% level of significance ($p \leq 0.05$).

***Data collection and data management plan***. A central database will be located and managed at the Institute for Physical Activity and Nutrition (managed by Dr Hannah Thomas) and all data retained for a minimum of 15 years from the date of final publication, as per regular policy for clinical studies. Electronic data will be stored and backed-up in a secure manner on password-protected electronic storage at Deakin University using a secure internet link and dedicated software including Syncplicity and a School shared networked drive (only named research investigators have access to these storage locations), in accordance with Deakin University's storage recommendations. Hard copy files and data will be stored in locked filing cabinets at Deakin University (Dr Hannah Thomas's office).

## 8. Data review and management

***Site monitoring.*** A variety of original documents, data, and records will be considered as source documents in this study. The Investigators will ensure reliability, quality, integrity, and traceability of all data sources and records for potential HREC and TGA inspections. Any electronic source data processes will be clearly validated to ensure accurate, legible, original, attributable (e.g., username and password), and contemporaneously entered and meet regulatory requirements for record keeping and retention.

The study may be subject to audit by regulatory authorities. If such an audit occurs, the Investigator will allow access to participant records.

***Data collection, data management and data sharing.*** Case report forms (CRFs) in paper form will organise and summarise all pertinent data for this study. The CRFs will be available for inspection by monitors before, during, and after study completion. Source verification will ensure that data recorded is not missing, inconsistent or implausible.

Each participant will be given a unique code upon enrolment which will be used to code all data for storage in a re-identifiable format. All samples and data collected will use this unique code. A master excel spreadsheet containing the code/key to re-identify data will be managed by the Principal Investigator and hosted on a secure School networked drive that only the research investigators named on the ethics application have access to (NOTE: all researcher investigators will be given access in the event that the Principal Investigator is unavailable and re-identification of data is required for safety reasons). It is necessary to be able to re-identify the source data during circumstances where participants have abnormal test results that require the study team to contact the participant, and recommend they contact their GP/doctor for follow-up. Statistical analysis will be performed on non-identifiable data and material (e.g., raw values that are not associated with a participant code or identifiable information will be copied into the statistical package software). Non-identifiable data (raw values) will be used for the dissemination of all findings and public facing documents (e.g., scientific publications, conferences, and reports).

All study data will be collected by the Investigators and recorded on source documents. The data will be directly recorded on or transcribed to study-specific CRFs from the source documents, cross-referenced and double-checked. Note that all questionnaires completed by participants will act as both source document and CRF (i.e., no transcribing of answers to a separate form).

Electronic data will be stored and backed-up in a secure manner on password-protected electronic storage at Deakin University. The primary electronic storage system used is a shared School network drive that only investigators of the research team have access to. Data will be backed up and stored to a project folder using Syncplicity which only the research investigators will have access to. Hard copy data may be collected if electronic collection is unavailable (e.g., questionnaires which will be coded/re-identifiable). Hard copy documents will be stored in locked filing cabinets at Deakin University (Dr Hannah Thomas's office) in accordance with site-specific security policies until they can be destroyed after the retention period (15 years from the date of final publication). Basic identifiable information (e.g., name, data of birth) will be associated with a re-identifiable code and securely stored on the previously mentioned master excel spreadsheet which will be managed by the Principal Investigator. All other data collection will use the re-identifiable code to protect participants' privacy and maintain blinding. Study findings will be presented at scientific conferences and submitted for publication in peer-reviewed scientific journals using non-identifiable data (raw values). No identifiable data will be included in any outputs. Research staff may be granted access to identifiable information as required for the purposes of contacting participants, data entry verification, analysis,

auditing, or other purposes, which will need to be approved by the data manager (the Principal Investigator).

Participants will retain ownership of their individual data. The Coordinating Centre (Deakin University) will hold responsibility for storing, protecting, and retrieving study data and hold responsibility for the safe guardianship and use of the data. Participant will be able to request access to their data, and corrections to personal information, via the Principal Investigator. Corrections to outcome data will only be permitted in extenuating circumstances with approval from the Principal Investigator, to prevent introducing bias as a result of retrospective data modification. Access to study information will be controlled by the Principal Investigator and restricted to members of the research team. All investigators will be able to request access to the full study dataset. The Principal Investigator may approve access for other individuals if required for regulatory or auditing purposes. Participants will be able to request summaries of their individual results at the end of their participation period, and/or the overall study findings after the study has been completed. Information to participants will be directly provided to them in a re-identifiable (coded) format to maintain participant confidentiality.

All participant data will be securely archived at Deakin University after completion of the trial. Archived data will be retained for at least 15 years from the date of final publication, in line with Deakin University data management policy for research involving clinical trials. Within this period no records will be destroyed without approval from the Principal Investigator. After that period hard copy data will be securely destroyed. Electronic data will be digitally deleted. If the Coordinating Principal Investigator or any Coinvestigator withdraws from the study (e.g., relocation, retirement), any records they hold will be transferred to a mutually agreed designee (e.g., another Co-investigator). Notice of such transfer will be given in writing to the Deakin University Human Research Ethics Committee.

Non-identifiable data may be made available on publicly available databases to comply with academic journal data sharing policy. In this case, all publicly available data will be non-identifiable (raw values not associated with a code or identifiable information). The potential sharing of non-identifiable data has been described in the PLS in the following sentences: “It is anticipated that the results of this research project will be published and/or presented in a variety of forums. In any publication and/or presentation, information will be provided in such a way that you cannot be identified, except with your permission. All data will be presented as the average of the group or non-identifiable raw values. It is possible that data may be presented in a publicly available database, as per policy of many academic journals. It is important to know that the information in this study will only be used in ways that will not reveal who you are. You will not be identified in any publication from this study or in any data files shared with other researchers. Your participation in this study is confidential.”

***Quality control.*** Study personnel will implement and maintain quality control procedures to ensure that the study is conducted, and that the data are generated, documented, and reported in compliance with the protocol, good clinical practice, and applicable regulatory requirements.

The study records will be retained at the site for 15 years and measures will be taken to prevent accidental or premature destruction of these documents.

## 9. Risks associated with participation

***Participant privacy during ultrasound assessment of the heart:*** In some cases, participants (predominantly females) will be asked to wear a loose research gown instead of a shirt to assist with access to the chest and obtaining a clear image of the heart. This is common practice and often necessary when imaging the heart.

***Blood sampling:*** Risks include some pain and discomfort upon cannula insertion. Risks also include vasovagal episodes, bleeding, bruising, thrombophlebitis and infection.

***Maximal aerobic exercise testing:*** Risks include muscle soreness, musculoskeletal injury, vasovagal episodes, dizziness, syncope, heart attack and sudden death.

***Hypertension:*** It is possible that participants may experience hypertension during or following exercise.

***Definity contrast agent infusion:*** A small number of people (8.4% of people) have side-effects during the infusion of the contrast agent (Definity) during ultrasound imaging. The most common of these side-effects include: back pain (1.2% of people), chest pain (0.8%), headache (2.3%), dizziness (0.6%), nausea (1.0%), flushing (1.1%). Serious cardiopulmonary or allergic reactions are incredibly uncommon and usually occur within 30 minutes of administration. Associate Professor Keske has been working with the contrast agent (Definity) for 20 years and has completed >500 infusions in people without serious adverse events. Risk of infection is very low given the contrast is suitable for intravenous infusion and we use sterile equipment and aseptic techniques. Participants will remain in the laboratory until at least 30 minutes after the last Definity infusion.

***Glutathione infusion.***
Intravenous infusion of glutathione can cause rare side effects such as anaphylaxis, infection, headache, dizziness, and nausea.

***Adverse events (AE)***
All AEs will be recorded in the study case report form and include the following information:

- Nature (brief description);
- Time and date of onset;
- Time and date of resolution;
- Maximum intensity (mild, moderate, severe);
- Seriousness;
- Treatment given;
- Relationship (causality) to the test product (scale: not related, possibly related, probably related, and related);
- Action taken; and
- Outcome

***Rating scale for maximum intensity***
Maximum intensity of an AE is the accumulated degree of discomfort, or if the AE is life threatening. Intensity should be assessed according to the following definitions:

| | |
|---|---|
| *Mild:* | Awareness of signs or symptoms, but these are easily tolerated (acceptable). |
| *Moderate:* | Discomfort enough to interfere with usual activity (disturbing). |
| *Severe:* | Incapacity to work or to do usual activity (unacceptable). |

***Rating scale for causality***
The Investigator will be asked to assess the causal relationship to the study drug according to the following classifications:

| | |
|---|---|
| *Not related:* | Time relationship with study is non-existent or doubtful, or other factors, certain or probable, to have been causative. |
| *Possible:* | Time relationship with study exists. Other possible causative factors may exist (e.g., concurrent disease or concomitant medication). |
| *Probable:* | Time relationship with study exists. No other possible causative factors may exist (not reasonably explained by the participant's known clinical state or concomitant |

medication). Recurrence of symptoms on rechallenge (if performed) has occurred. A specific laboratory investigation (if performed) has confirmed the relationship.

*Definite:* Those events for which there is no shadow of doubt that they are a consequence of the study. It is likely that such events will be widely documented and generally accepted as having association with the test product or that they reoccurred after rechallenge (if performed).

***Serious adverse events (SAEs)***

A SAE or serious adverse reaction is any untoward medical occurrence that:

- Is fatal or life-threatening.
- Results in persistent or significant disability/incapacity.
- Requires inpatient hospitalisation or prolongation of existing hospitalisation.
- Results in a congenital anomaly/birth defect.
- Is an important medical event that may jeopardise the participant or may require medical intervention to prevent one of the above-listed outcomes.

All SAEs will be immediately reported to the Deakin University Human Ethics Committee.

## 10.How will the risks be minimised?

***Screening prior to participation.*** Screening will minimise risks by excluding those with contraindications to the conditions and techniques carried out in this study.

***Trained and experienced research staff.*** All testing sessions will be attended by at least one staff member who has first aid and advanced CPR training (note: two staff members will always be present). Prior to each testing visit the research team will perform a crash cart check to confirm that all resuscitation equipment (including defibrillator) is available and working. If a participant experiences chest pain, suspected heart attack, or any other condition warranting immediate action, the research team will commence standard first aid and advanced CPR training procedures as per their training which includes 1) calling 000 immediately; 2) perform CPR and use a defibrillator if necessary; 3) administer supplemental oxygen if necessary; and 4) continue CPR and/or first aid procedures until paramedics arrive. Participants will be referred to their general practitioner for other non-emergency health issues.

***Maximal aerobic exercise testing.*** High-intensity exercise has previously been used in patients with PAD and/or chronic heart failure without serious adverse effects [29, 40-43]. As such, the exercise test is not expected to directly result in any adverse effects. However, all physical activity has some risks. Although rare, the two common cardiac risks include myocardial ischemia and different forms of arrhythmias. It is estimated that for adults without existing heart disease, the risk of a cardiac event or complication ranges between 1 in 400 000 – 800 000 hours of exercise. For patients with existing heart disease, an event can occur an average of once in 62 000 hours [44]. To minimise the risk of maximal exercise testing, all exercise tests will be completed with an ECG fitted to detect abnormal heart rhythms and each session will be supervised by experienced exercise physiologists (members of the research team who have extensive experience conducting exercise tests in clinical populations). If an abnormal heart rhythm or blood pressure is detected the test will be stopped and the participant will be asked to see a cardiologist for assessment and approval prior to continuing with the study. In addition, to reduce risk of falls and increase participant comfort, a minimum of two experienced researchers will be present during every exercise test. The exercise test will be terminated immediately if any of the following criteria are present:

- Participant wishes to stop.
- Experiences chest pain, severe shortness of breath or any other pain related to or caused by exercise.

• Abnormally high heart rate relative to exercise-intensity, abnormal heart rhythm, abnormally high blood pressure, or other signs of metabolic, cardio-respiratory or thermo-regulatory distress.

If a medical disorder is suspected at any time throughout the study, participation will be ceased immediately, and the participant will be referred to a cardiologist for assessment and approval prior to continuing with the study.

***Participant privacy during ultrasound assessment of the heart.*** The research laboratory has curtains and dividers to ensure privacy for the participant. The process behind imaging the heart will be comprehensively discussed with the participant prior to enrolment in the project (i.e., during the informed consent phase). All efforts are made to ensure privacy is maintained for the participant at all times. This procedure, and what is involved, is discussed with participants during the informed consent and screening phase, and during the familiarisation sessions.

***Management of risks associated with blood sampling.*** The use of sterile and disposable catheters, syringes, gloves and gauze will markedly reduce the possibility of infection. The use of staff qualified and experienced in aseptic venepuncture and cannulation techniques will reduce the likelihood of bruising and infection. Sterile techniques and infection control procedures will be used at all times.

***Management of risks associated with L-glutathione infusion.*** Glutathione is one of the body's major antioxidant defence systems. It is a water-soluble molecule synthesised endogenously from the three amino acids glycine, cysteine and glutamic acid, which are all found naturally in the food we eat. Glutathione is classified as a prescription drug (Class S4) and is predominantly used in research due to its safe and effective use as an antioxidant / free radical scavenger (i.e., it is predominantly used to decrease oxidative stress) [45]. The half-life of glutathione following intravenous infusion has been calculated to be around 15 minutes [46]. Therefore, its bioactivity is transient and decreases rapidly after cessation of infusion. Intravenous infusion of glutathione has previously been performed in healthy humans and clinical populations [20, 21, 45-49], with no reported adverse events or side-effects with short-term transient use (i.e., single infusions). The glutathione dose varies between studies depending on the IV infusion rate and the infusion duration, and whether it is prescribed on participant body weight [20, 21, 45-48]. The infusion rate we have elected for (15 mg/min over 2.5 hours [approximately 2,250 mg]) has previously been used in healthy adults to decrease oxidative stress and is well tolerated [20]. Similar absolute doses have been infused in humans (2,600 - 4,700 mg; depending on body weight) over the much shorter infusion duration of 15 minutes (up to 313 mg/min; i.e., a much higher effective dose administered than the proposed study), without any reported adverse effects [46]. Importantly, we have previously performed several infusions in healthy adults at the dose of 15 mg/min, over a number of hours (up to 6 hours) DUHREC: 2019-202, without any adverse effects.

***Management of risk associated with the Definity contrast agent infusions.*** The infusion of Definity contrast agent is considered safe for use in both healthy and clinical populations including outpatients, hospitalised patients (including the critically ill), patients undergoing stress echocardiography, and patients with pulmonary hypertension [50, 51]. Nevertheless, under rare circumstances serious allergic and anaphylactoid reactions may occur immediately after contrast agent infusion (incidence is estimated to be at 0.009% and 0.004%, respectively [50]). As such, all contrast agent infusions at Deakin University are supervised by staff with the necessary cardiopulmonary resuscitation skills. Furthermore, to minimise contrast agent infusion adverse effects and prevent overdose the following procedures will be in place:

- As per standard operating procedures for injectables into humans, all substances (contrast agent and saline) to be infused/injected will be recorded for their expiration date and crosschecked by a minimum of 2 researchers prior to infusion/injection.

- Use of a dedicated syringe pump for the contrast agent with the infusion rate set at a constant, predetermined rate.
- Only named research investigators will make up the contrast agent solution to be infused, using only ampoules of the Therapeutic Goods Administration approved commercially available contrast agent (Definity, Lantheus Medical Imaging).
- A maximum of 3 ml of Definity agent will be infused in any one day as per previous studies conducted at Deakin [15, 17].

All contrast agent infusions will be overseen by cardiopulmonary resuscitation personnel. Resuscitation equipment will be readily available, and cross-checked, prior to commencing infusions. In the unlikely event of a serious reaction, we will contact emergency services following standard laboratory procedures for an adverse event and/or emergency, however this is incredibly rare.

***Sourcing of glutathione.*** Glutathione will be sourced and compounded by YourCompoundingSolution which specialises in compounded sterile preparations (CSP) and uses TGA approved facilities that adhere to current PSA and USP (797) standards (https://www.yoursolutioncompounding.com.au). This company has a new clinic in Hawthorn, 3123, and have the facilities to conduct their own sterility testing. Although adverse events have been reported in Australia for intravenous infusion of L-glutathione, these cases were linked to contamination of the glutathione compound, rather than the techniques, doses, or the bioactivity of the compound itself, and were prescribed and performed in a clinic rather than a research environment [52]. All staff involved in the sterile compounding process of glutathione are trained and accredited in the preparation of aseptic compounds and are annually re-certified for proficiency. Prescriptions are checked three times by pharmacists during the compounding process, with the last step after preparation for colour and/or clarity, consistency, packaging integrity, and readability of label instructions, to ensure quality control.

**14. For research involving an unapproved therapeutic good (such as a drug, device or biological):**
14.1 Does this project involve an unapproved therapeutic good requiring a Clinical Trial Notification (CTN)? (See the Clinical Trials webpage for more information about CTNs)
☐ Yes – go to the next question.
☒ No – skip to Section 15 (results, outcomes and future plans)

14.2 Is Deakin intended to be the Sponsor?
☐ Yes – go to the next question
☐ No – skip to Section 15 (results, outcomes and future plans)

14.3 If Deakin is intended to be the Sponsor and the research requires a Clinical Trial Notification (CTN), has the CTN, Clinical Trial Sponsorship Request Form and Protocol been submitted to research-integrity@deakin.edu.au for assessment?

☐ Yes – assessment completed and the CTN must now be submitted to the Therapeutic Goods Administration (TGA) by Deakin (as Sponsor). Please attach evidence of assessment and the CTN form. You will be contacted by the Human Research Ethics Office regarding submission of the CTN to the TGA.

If not, please submit the draft CTN, Clinical Trial Sponsorship Request Form and Protocol to research-integrity@deakin.edu.au for assessment before submitting this application to DUHREC. See the Clinical Trials webpage for further information. The Clinical Trial Sponsorship Request Form can be requested by contacting research-integrity@deaknin.edu.au.

14.4 What is/are the drug(s) and/or device(s):

- Approved name
- Trade name (if any)
- Manufacturer
- Supplier of drug/device (e.g. manufacturer/pharmacy)
- Approved therapeutic indication, dosage/duration in Australia
- Believed mode of action
- Dosage regimen
- Mode of excretion
- Known adverse events
- Known contra-indications or warnings
- If arrangements have been made for a Pharmacy Department to receive or dispense the drugs involved in this project, explain how the drugs will be received and dispensed for the purposes of the research project

**DECLARATION AND SIGNATURES**

I/We, the undersigned declare that the information supplied in this application (including the attached original application) is true and accurate to the best of my/our knowledge.

I/We the undersigned have read the *National Statement on Ethical Conduct in Human Research* and accept responsibility for the conduct of the project detailed in this application in accordance with the principles contained in the Statement and any other conditions laid down by Deakin University Human Research Ethics Committee.

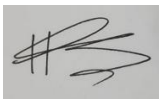
I/We the undersigned, declare that where the research project may involve contact with a child or young person under the age of 18, I/we have a current Working with Children Check.

**Principal investigator**

Name: **Dr Hannah Thomas**

Human Ethics Quiz (please complete the appropriate box below):

☒ successfully completed the Human Ethics Quiz (compulsory for Deakin staff and students)
☐ exempt from completion of the Quiz due to prior inclusion on an ethics application at Deakin. *Please indicate HEAG or DUHREC Project ID:* Click or tap here to enter text.

Signature:  Date: **25/05/2022**

**Associate investigators***

Name: **Dr Lewan Parker**

Affiliation (please select from the drop-down list by clicking on 'Choose an item'): Choose an item.

Human Ethics Quiz (please complete the appropriate box below):

☒ successfully completed the Human Ethics Quiz (compulsory for Deakin staff and students)
☐ exempt from completion of the Quiz due to prior inclusion on an ethics application at Deakin. *Please indicate HEAG or DUHREC Project ID:* Click or tap here to enter text.
☐ external researcher (exempt from completing the Quiz)

Signature:  Date: **25/05/2022**

Name: **A/Prof Michelle Keske**

Affiliation (please select from the drop-down list by clicking on 'Choose an item'): Choose an item.

Human Ethics Quiz (please complete the appropriate box below):

☒ successfully completed the Human Ethics Quiz (compulsory for Deakin staff and students)
☐ exempt from completion of the Quiz due to prior inclusion on an ethics application at Deakin. *Please indicate HEAG or DUHREC Project ID:* Click or tap here to enter text.
☐ external researcher (exempt from completing the Quiz)

Signature: Michelle A Keske Date: **26/05/2022**

Name: **Dr Andrew Garnham**

Affiliation (please select from the drop-down list by clicking on 'Choose an item'): Choose an item.

Human Ethics Quiz (please complete the appropriate box below):

☐ successfully completed the Human Ethics Quiz (compulsory for Deakin staff and students)
☒ exempt from completion of the Quiz due to prior inclusion on an ethics application at Deakin. *Please indicate DUHREC Project ID:* 2021-048 and 2019-426
☐ external researcher (exempt from completing the Quiz)

Signature: Date: **26/05/2022**

**Please copy and paste the above for each additional associate investigator.**

*****All research staff involved in the project must sign the project description/protocol. Please add additional signatures blocks as required.

**ACKNOWLEDGMENT OF HEAD OF SCHOOL/DIRECTOR OF RESEARCH****

I, the undersigned, acknowledge that the School/Faculty/Institute has considered and approved the academic worth of the project described in this application.

Name: **Prof Glenn D Wadley**

Signature: Date: **27/05/2022**

**If the Head of School (or similar) is also a member of the research or supervisory team, a more senior member of University staff e.g. Dean or Associate Dean (Research), must sign the project as authorising officer.

A Project Description is a **mandatory** component of a submission using the Human Research Ethics Application (HREA).

Please submit all documents via direct email to <research-ethics@deakin.edu.au>.

Deakin University is collecting your personal information on this form for the primary purpose of processing your human research ethics application. It will also use this information for monitoring your compliance with the approved protocol. For these purposes Deakin may also provide this information to potential research participants, past or current research participants, or other interested parties in your research. You are not required to provide the information requested, however if the information is not provided, Deakin may not be able to process your ethics application. Deakin manages personal information it holds, including requests by individuals for access to their personal information, in accordance with the Privacy and Data Protection Act 2014 (Vic). Deakin's Privacy Policy may be viewed on Deakin's Policy Library. Information on privacy at Deakin is available at http://www.deakin.edu.au/footer/privacy. Questions about privacy may be directed to the Privacy Officer on (03) 5227 8524 or by email to privacy@deakin.edu.au.

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