Study Protocol

Mechanistic Insights into changes in peripheral blood flow following intermittent negative pressure.

Study Acronym	PAD Protocol
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Chief Investigator Principal Investigator	Professor Faisel Khan Jody McIntosh
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Protocol approval

Mechanistic Insights into changes in Peripheral Blood flow following intermittent negative pressure.

Signatures

By signing this document I am confirming that I have read, understood and approve the protocol for the above study.

Prof F Khan	Signature _____	Date_____
Chief Investigator		
Jody McIntosh	Signature _____	Date_____
Principle Investigator		

List of abbreviations

CI	Chief Investigator
CRF	Case Report Form
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
GCP	Good Clinical Practice
GP	General Practitioner
HV	Healthy Volunteer
ICF	Informed Consent Form
IHD	Ischaemic Heart Disease
ISF	Investigator Site File
KO	Knock Out
LPLV	Last Patient Last Visit
NHS	National Health Service
NO	Nitric Oxide
PBMCs	Peripheral Blood Mononuclear Cells
PIS	Participant Information Sheet
PAD	Peripheral Arterial Disease
PVD	Peripheral Vascular Disease

PWV	Pulse Wave Velocity
ABPI	Ankle-Brachial Pressure Index
QC	Quality Control
REC	Research Ethics Committee
ROS	Reactive Oxygen Species
SMF	Study Master File
TASC	Tayside Academic Sciences Centre
INP	Intermittent Negative Pressure
FMD	Flow Mediated Dilation
ACh	Acetylcholine
SNP	Sodium Nitroprusside

Summary

Atherosclerosis occurs when blood supply to the lower limbs is restricted upon accumulation of fat in the arteries. The initial symptom is pain in lower limbs followed by hypoxia and ulceration. The literature has indicated that intermittent negative pressure (INP) can be used to reduce the pain and facilitate wound healing. INP is a non-invasive technique that aims to increase blood flow in lower limbs and foot but there is no evidence to prove these findings. In this PhD studentship, we aim to investigate the underlying mechanisms that are involved in changes in blood flow following application of INP. The Flow-Ox 'boot' will be used to apply INP for periods ranging from 4-8 weeks, for an hour twice per day, to the lower limbs to determine the effects of INP on vascular function and blood flow. Specifically, vascular tests such as assessments of endothelial function (laser perfusion imaging with iontophoresis, flow mediated dilatation of the brachial artery), arterial stiffness (pulse wave analysis), ankle-brachial pressure index (ABPI) and blood borne metabolic and inflammatory markers will be performed before and after INP application. A pain chart will be employed before and after INP application to determine whether there is any change in perception of pain felt by individuals who suffer from pain associated with lower limb vascular disease.

INTRODUCTION

Background

Peripheral arterial disease (PAD) is a vascular condition that causes lower limb pain or discomfort (1). Diseases that facilitate stenosis or occlusion of lower limb arteries result in PAD. Atherosclerosis is a common cause for PAD as it results in the development of atherosclerotic plaque which restricts the blood supply to the lower limbs resulting in decreased oxygen transport and poor tissue perfusion (1). This creates a hypoxic environment in the lower limbs and leads to the perception of pain (1). The level of tissue damage is dependent upon the size of the obstruction and restriction of blood supply (1).

PAD attenuates both life expectancy and the quality of life as it increases the risk of cardiovascular diseases (CVD) such as myocardial infarction and stroke (1). Evidently PAD and CVD share common risk factors which include; age, smoking, dyslipidaemia, hypertension and diabetes mellitus (2). The risk of CVD related mortality can be reduced by risk factors modifications; prescribing antiplatelet therapy, ACE inhibitors and statins to PAD patients (1). Although, PAD has many clinical presentations it can be categorised into three classes: intermittent claudication (IC), critical limb ischaemia (CLI) and asymptomatic disease (3). IC is the typical presenting symptom and is usually perceived as muscle fatigue, cramps or pain in the lower limbs (1)(3). The characteristic symptom of claudication is elevated pain upon exercise and its resolution by sufficient rest (1). In fewer cases, patients may also experience CLI, which is clinically recognised as rest pain and defined phenotypically by either ulceration and gangrene of the foot, or physiologically by a toe systolic blood pressure of <30mmHg or an ankle systolic blood pressure of <50mmHg (1)(3).

All first line treatment modalities for PAD aim to impede diseases progression and augment blood flow (4). These include behavioral modifications that aid risk factors regression and enhance exercise performance (4). If behavioral alterations fail to deliver expected results, an alternative line of treatment is revascularisation through open surgery or endovascular surgery (4). While the innovation of endovascular techniques has transformed the field of vascular treatment in PAD, it has not changed the selection of individuals that are expected to benefit from revascularization and unsurprisingly this line of therapy is proven to be highly invasive and inadequate for many PAD patients (3)(4). There remains a need for novel treatment options that can help treat PAD in a non-invasive manner. Intermittent negative pressure (INP) is one such non-invasive technique that aims to increase blood flow in ischaemic limbs. Although the idea that air pressure can be regulated to increase distal circulation was first described by

Murray et al in mid-19th century, it has only recently been demonstrated in healthy volunteers to improve peripheral blood flow of the foot with minimal effect on central haemodynamics (4)(5). Likewise, many other case studies have highlighted increased skin temperature, wound healing and reduced pain perception in PAD patients when INP was applied. On the contrary employing a constant negative pressure has shown to elevate vascular resistance which causes a sequential fall in blood flow (5). One way in which vascular resistance is increased is due to the rise in venous pressure and the concurrent activation of the vasoconstrictor reflex that reduces blood flow in peripheral tissue (5). These findings illustrate that INP with brief oscillations of negative pressure applied to lower extremities can circumvent the venoarteriolar reflex and increase peripheral blood flow (6). Additionally, a study by Sundby et al illustrated the beneficial effects of applying INP of -40mmHg for 10-30 seconds to lower limb of healthy volunteers as it led to an increase in both the micro- and macrocirculation (5). This increase was shown to be INP-induced as baseline measurements where negative pressure was maintained on lower limbs for long periods without oscillation did not increase blood flow in this region (5). No known side effects have been reported. Sundby et al also showed the positive effects of INP on the ischaemic PAD patients with an increase in the blood flow velocity and skin perfusion. These positive results are thought to be due to the INP-induced shear stress response. It is suggested that INP plays a role in regulating vascular tone through endothelial cells, although direct evidence for this is lacking. Prostacyclin and endothelium-derived hyperpolarising factor (EDHF) mediators are thought to be involved in the positive effects on blood flow velocity. Endothelium-dependent nitric oxide (NO) release from endothelial cells could be having a significant effect after INP application in PAD patients. Although it is known that INP mediates increased cutaneous and arterial blood flow in healthy volunteers as well as in PAD patients, the mechanism underlying these effects remain elusive in the macro- and microcirculation of the foot.

STUDY OBJECTIVES

The aim of the present PhD project is to better understand the potential physiological mechanisms involved in regulating changes in blood flow following application of INP.

Primary Objective:

- Investigate changes in blood flow and the relationship between changes in markers of vascular function (endothelial function & arterial stiffness) and application of INP.

Secondary Objectives:

- Determine whether any changes in blood flow are associated with corresponding changes in blood-borne markers of endothelial activation/damage and inflammatory and oxidative stress markers.
- Determine the effects of INP on changes in markers of vascular function after acute and prolonged application.
- Determine whether the changes are confined to the local application sites or whether INP applied to one extremity exerts systemic effects on the vasculature.

To test our objectives, the specific aims are outlined below:

- Assess the changes in blood flow in the lower limbs using laser Doppler perfusion imaging before and after INP is applied. This will provide a good indication of the microcirculation. Iontophoresis of vasoactive chemicals (acetylcholine and sodium nitroprusside) will also be used to evaluate the role of endothelium-dependent and -independent mechanisms, respectively.
- Assessment of the systemic endothelial function will be measured using the flow-mediated vasodilatation (FMD) of the brachial artery. The change in FMD% will be a good indicator of the INP effect on macrovessels.
- Vascular assessment will also be recorded in the form of arterial stiffness. SpygmoCor will be used to record the peripheral pressure waveforms at carotid and femoral/radial sites in the body.
- Determine the ankle-brachial pressure index (ABPI) of PAD patients before and after INP is applied.
- Compare pain chart measurements before and after the INP procedure in patients experiencing pain associated with PAD.
- Assess levels of inflammatory and metabolic markers (E-selectin, I-CAM, VCAM, IL-1β, IL-6, IL-10, CRP, TNF-α, Nrf2 expression, malondialdehyde, reduced & oxidised glutathione) in the blood before and after INP.

The procedures outlined above, will be applied on the first visit before INP and following 1 session (to test acute effects) and following repeated sessions for up to 8 weeks to examine the effects of prolonged applications of INP to determine the duration for optimal improvement in vascular function.

STUDY DESIGN

Two groups will be evaluated in this PhD study – healthy volunteers and PAD patients (figure 1). This will be a single-blinded placebo controlled cohort study. All healthy volunteers will receive active -40mmHg

INP while PAD patients will receive either an active device (-40mmHg INP) or placebo device (-10mmHg INP).

Randomisation

This will be a single-blinded study. The patients will not know whether they have been assigned to the active or placebo device. The researcher will know which device a patient has been assigned to. 80 PAD patients will be recruited in total. The first 20 PAD patients will not be randomised and will be assigned the active device. The subsequent 60 PAD patients will be randomised on a 2:1 basis with 40 patients assigned the active device and 20 assigned placebo. This will allow for more mechanistic studies to be carried out on patients receiving active -40mmHg INP. Patients will be randomised to either active or placebo using an online randomisation programme.

Patients will be told which device they were assigned to at the end of the study.

At the start of the study, eligible patients with an ABPI <0.9 will be approached in wards and clinics by the study team and vascular surgery team. Patients will be given an invitation letter along with an information sheet and reply slip. The study team will offer the patients INP and the patients will be trained to use the Flow-Ox themselves.

***Group 1*.** 40 healthy subjects will be approached by e-mail and posters at Ninewells. Healthy volunteers will be studied to explore the effects of acute application (1 session) and repeated application (daily for up to 5 days) of INP on measurements described above. Importantly for the purposes of this PhD project, this group of subjects will allow us to explore the mechanistic effects of INP on normal physiology.

Group 2. 80 patients with PAD will be recruited from in-ward patients and from outpatient vascular clinics. Patients will be recruited by the Vascular Surgery team lead by the Consultant Vascular Surgeon who is the second Academic Supervisor of the PhD student. Patients will be randomised to active INP device (-40mmHg) or placebo INP device (-10mmHg). 20 patients in total will receive the placebo device and 60 will receive the active device. The effects of INP on the measurements described above will be made at baseline (before application of INP), following 1 session of INP (acute effects) and following 4-8 weeks of daily INP. As this is a PhD project, not all patients will undergo 8 weeks' of INP. Based on the initial studies of the shorter 4-week duration, we will be able to examine how many patients will be tested for 8 weeks.

Before application of INP, anthropometric baseline measurements (height, weight) will be taken from all participants at Level 7, Division of Systems Medicine, Ninewells Hospital. For patients, medical notes will be reviewed for clinical characteristic including information on past medical history, drug therapy and blood biochemistry.

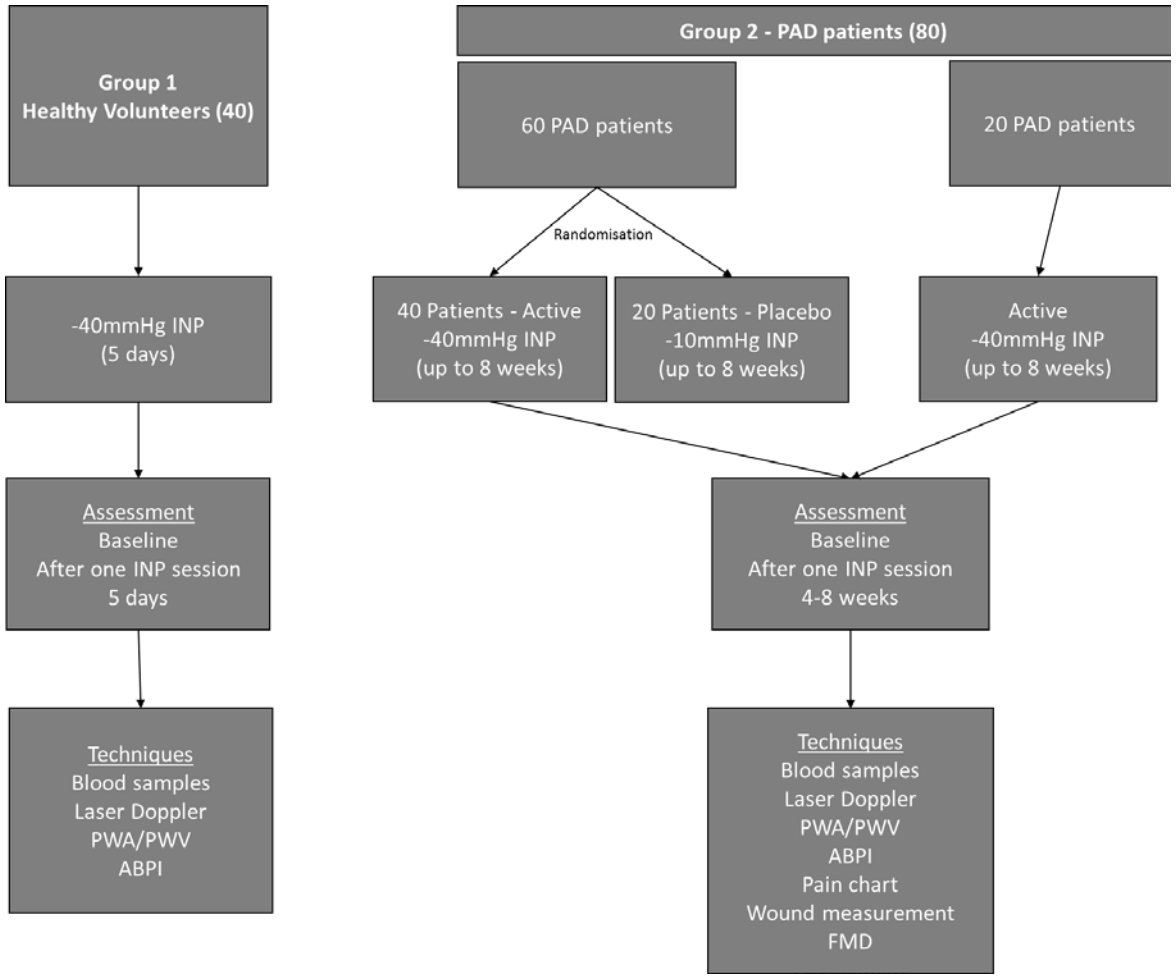

Figure 1:Flow Chart of study design and measurement techniques.

FLOW-Ox System:

Application of INP will be made using the Flow-Ox machine, a CE marked device provided by Otivio as a gift (Otivio AS, Oslo, Norway) (Figure 1). Participants will be asked to be comfortably clothed and seated (with a 130-degree angle between their hip and knee joint) for 20 minutes prior to the start of the experiment. They will be asked to place a leg into the Flow-Ox chamber which is a molded polyethene

pressure chamber that is designed specifically in a boot shape to fit the leg. The other leg will form the control and will be placed outside the pressure chamber in a normal atmospheric pressure. The internal padding of the boot will allow insertion of the leg without pressure points on the skin. The boot will be sealed below the knee with a thermoplastic elastomer. This will help to apply the negative pressure to the lower limb. The pressure will be continuously monitored by a pressure transducer attached within the boot. Firstly, a two-five-minute baseline recording without any pressure change (atmospheric pressure, 760mmHg) will be recorded. Consecutively INP will be applied for one hour, twice per day, using a cyclical protocol that comprises of 10 seconds of -40mmHg negative pressure and 7 seconds of atmospheric pressure (as outlined by Sundby et al 2016) (5). The placebo device delivers negative pressure of -10mmHg by the same cyclical protocol as the active device but does not alter blood flow. For studying the longer-term effects of INP application, participants will be trained to use the Flow-Ox machine in their homes and will return to the hospital for follow-up assessments at 4 or 8 weeks. Patients will be contacted weekly (and where appropriate visited at their homes) to ensure no problems are encountered and to encourage adherence to the study.

Figure 2: Illustration of patient set up with the Flow-ox boot.

Blood Samples

40ml of blood will be obtained from each study subject for serum markers of inflammatory and oxidative stress. Serum will be analysed using Bio-Plex® Precision Prokits™ (BIO-RAD laboratories) for IL-1β, IL-6, IL-10, TNF-α, CRP, E-selectin, sICAM-1. Peripheral blood mononuclear cells (PBMC) will be extracted for analysis of expression of Nrf2 by qRT-PCR analysis, measuring the expression of Nrf2-target genes. The genes to be analysed will include those encoding GCLC, GCLM, GSR1 and SLC7A11 (synthesis and regeneration of GSH), TXNRD1 and SRXN1 (reduction of protein thiols), GPX2, HMOX1, PRXD1 and PRDX6 (ROS scavenging and stress biomarkers), AKR1B10, AKR1C1, AKR1C2 and NQO1 (detoxification of oxidation products). Western blotting will be performed to measure the protein levels of all Nrf2-target genes that are expressed in PBMCs as a means of corroborating qRT-PCR results.

The extent of oxidative stress will be estimated by measuring reactive oxygen species (ROS) using 2',7'-dichlorodihydrofluorescein, malondialdehyde by an LC-MS assay, and reduced glutathione (GSH) and oxidised glutathione (GSSG) by a standard LC-MS assay.

The surplus blood samples will be stored for 15 years at -80°C in the department, Division of Systems Medicine at Ninewells Hospital, and will be registered with Tayside Biorepository.

Laser Doppler Imaging

Laser Doppler imagining (using the Moor Instrument FLPI system) will be used to measure microvascular endothelial functions and blood perfusion of the lower limbs and arm. This test will be a good measure of how the lower limb circulation changes after INP application and if any systemic changes in microcirculation occur in the arm. The laser will be mounted on an adjustable arm attached to a portable stand. This will be positioned 20-30 cm above the skin to capture the image. The camera records blood flow in the superficial microvessels (arterioles, venules and capillaries) up to 1mm deep in skin. Repeated scans will be obtained and the perfusion calculated in arbitrary units. The microvessels of the lower limb will be stimulated using iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) to assess endothelium-dependent and –independent mechanisms, respectively.

At each assessment, wounds will be photographed using the laser Doppler imager and measured using appropriate software (eg. Photoshop).

Flow-Mediated Dilatation

This test assesses endothelial function of the macrocirculation of the (brachial artery) through flow mediated changes. This test involves using a high resolution ultrasound probe (5-8 MHz) to measure changes in the diameter of brachial artery following 5-minutes of ischaemia applied to the forearm using a blood pressure cuff inflated above systolic blood pressure (normally 200mmHg). The measurement of brachial artery diameter will be taken on three occasions; baseline, during occlusion and after deflation. This test is not anticipated to have any risk to the participants.

A spray of glyceryl trinitrate will be given under the tongue to help assess vascular function. Glyceryl trinitrate is a medicine that is normally used in the treatment of angina (a feeling of tightness in the chest), and can cause a mild headache. Blood pressure will be checked before GTN administration. If systolic is below 100 mmHg, GTN will not be given to a participant.

Pain Chart

PAD patients, but not healthy volunteers, will be asked to fill the pain chart both before and after they use the Flow-Ox machine. In this form they will be asked to numerically rate their pain on a scale of 0-10 (ranging from no pain (0) to the worst pain they have ever felt (10)).

ABPI Monitoring

Ankle brachial pressure index (ABPI) is determined by measuring continues systolic pressure waves at the brachial, dorsalis pedis and posterior tibial artery using a handheld 8 MHz Doppler blood velocity detector.

Arterial Stiffness (Pulse Wave Velocity and Pulse Wave Analysis)

In this non-invasive test arterial stiffness will be assessed. This test gives information of the arterial pressures that represents the systemic arterial stiffness which measures aortic pulse wave velocity and augmentation index. The participant will lie on a bed and acclimatise for 10 minutes after which blood pressure will be measured in triplicate. The waveforms of the artery from the volunteer will be recorded at the wrist (radial artery), neck (carotid artery) and groin (femoral artery) with a micro manometer using the SphygmoCor PWV and PWA system. Augmentation index, heart rate and carotid-to-femoral pulse wave velocity will be calculated by using this software. This test carries no risks for the participant health.

STUDY POPULATION

Number of Participants

This study will recruit a total of 120 participants in total (80 PAD patients and 40 healthy volunteers).

Inclusion Criteria

PAD patients:

- Age ≥ 18 years
- At the vascular outpatient clinics or admitted in ward with PAD.
- ABPI <0.9

Healthy Volunteers:

- Age ≥ 18 years
- No current or previous significant cardiovascular illness
- Able to give written informed consent

Exclusion criteria

Healthy Volunteers:

- Positive medical history of:
  - ✓ Vascular diseases such as PAD, stroke, IHD, hypertension... etc.
  - ✓ Haematological conditions such as hypercoagulability, deep venous thrombosis... etc.,
- Alcohol excess
- Unable to give written informed consent
- Pregnant women

PAD patients:

- Unable to give written informed consent
- Patients with deep venous thrombosis
- Pregnant women

Individuals will not be enrolled to the study if they are participating in the clinical phase of another interventional study or have done so within the last 30 days. Individuals who are participating in the

follow-up phase of another interventional study, or who are enrolled in an observational study, will be co-enrolled where the CIs of each study agree that it is appropriate.

Pregnant women will not be included in the study. Pregnancy testing will be performed by research staff before the participant consents to taking part in the study.

PARTICIPANT SELECTION AND ENROLMENT

Identifying Participants

PAD patients: PAD patients will be identified by the Vascular Surgery team under the supervision of the Consultant Vascular Surgeon. The vascular clinical team will review the past medical history, drug therapy and biochemistry to ensure eligibility for the study. Patients with a diagnosis of PAD (ABPI <0.9 at rest) will be recruited into the study. They will approach PAD patients in the outpatient clinic or pre surgery hospital visit, with information about the study. Alternatively, the vascular clinical team may contact patients over the phone with information about the study then proceed to post a patient information sheet and reply slip to interested patients. Patients will be given an invitation letter, reply slip and PAD PIS. The PIS will have contact details for the patient to contact the study team with any questions or concerns about the study. The patients interested in the study will complete and return a reply slip directly to the CI/PI using a pre-paid envelope provided along with the PIS.

Healthy Volunteers: The initial approach to normal healthy subjects will take place amongst staff and students within the University of Dundee Medical School via posters displayed on notice boards around the Medical School at Ninewells and general e-mail circulations made through the School of Medicine Dean's Office. Interested volunteers will contact the research team either via email or telephone and PIS for healthy volunteers will be sent via the post or email. A minimum of 24 hours will be given prior to consent being obtained.

Consenting Participants

The consenting of participants from the 2 groups will be performed by the CI, PI or GCP trained delegated members of the study team competent in obtaining consent for research purposes.

PAD patients

The patients will be identified by the Vascular Surgery team who will approach them and deliver a PIS and reply slip in the outpatient clinic or pre surgery hospital visit. Patients who have read and understood the PIS will be consented by the CI/PI or delegated research team member. Consent will take place on the first visit at the Division of Systems Medicine, Ninewells Hospital just before the study procedure. Eligibility criteria will be checked before proceeding by the vascular team.

Healthy Volunteers

Healthy volunteers will be consented on the day of the study appointment, after being given adequate time to read the PIS and ask any questions (minimum of 24hours). Potential volunteers will be able to contact the researchers via contact details provided in the PIS. Consent will be obtained by the CI/PI or delegated research team member who is experienced in obtaining written consent for research purposes.

A copy of the signed Informed Consent Form (ICF) along with a copy of the PIS will be given to the study participant. The original signed consent form will be retained at the study site (filed in the TMF).

If new safety information results in significant changes to the study risk–benefit assessment, the Protocol, PIS and/or consent form will be reviewed, updated and amended as necessary. All participants, including those already being treated, will be informed of the new information, given a copy of the revised consent form and asked to re-consent if they choose to continue in the study.

Where a participant requests to speak with a physician from the study team the consent process will not be completed until the participant has spoken to the physician and had all their questions answered to their satisfaction.

For adults who lose capacity their previous wishes will remain legally binding and this will remain valid unless the protocol changes significantly. If this occurs and further consent is required from a participant who has lost capacity, the appropriate person will be asked for their consent. In all cases the CI or delegate will consult with carers and take note of any signs of objection or distress from the participant – the participant will be withdrawn if they raise objection. Where appropriate the participant will be withdrawn from any further clinical intervention and agreement will be sought from a carer to allow data collection.

The informed consent process will be conducted in compliance with TASC SOP07: Obtaining Informed Consent from Potential Participants in Clinical Research

Ineligible and Non-recruited Participants

The reason(s) for ineligibility will be explained to participants and any questions they have will be answered. They will be thanked for their participation in screening.

Withdrawal Procedures

Participants are free to withdraw from the study at any time. If at any time the participant formally withdraws his/her consent for future participation and disclosure of future information, no further evaluations will be performed and no additional data should be collected. Data collected before withdrawal will be retained and used in the study analysis, unless the participant requests otherwise. If participants withdraw during the visit, they will have the option to request that data already collected is not to be used in the study.

STUDY & SAFETY ASSESSMENTS

Patients will undergo their standard care as routine.

PAD patients

Research bloods (40 mls) will be obtained at the research site following informed patient consent and other study procedures such as pain chart, laser imaging, arterial stiffness and ABPI. This usually will occur on the 1st day in the designated research department, Division of Systems Medicine at Ninewells Hospital, and on two follow-up visits, after one INP session and 4 to 8 weeks after daily INP use. We do not envisage that any of these will interfere with their care or are likely to cause any harm. Nevertheless, any adverse events that may potentially be related to the study will be reported to the CI and clinical Vascular Team who will administer appropriate clinical action.

Healthy Volunteers

Research blood samples will be obtained at the research site following informed consent and other study procedures such as laser imaging, arterial stiffness and ABPI. This usually will occur on the first day in the designated research department, Division of Systems Medicine at Ninewells Hospital), and on two follow-up visits, after 1 INP application and again after up to 5 days daily INP.

DATA COLLECTION AND DATA MANAGEMENT

Data Collection

The patients will be given an identification number to ensure anonymity. Data collected will include gender, age, CHI number, previous medical history, medications, diagnosis, treatment, blood report findings, and personal history. This clinical data will be used in the analysis of results and will be used to correlate with the findings of the vascular assessments. The data will be collected by the research team on a paper Case Record Form (CRF), which will not hold any name or initials, with subsequent transcription into an Excel database. Electronic storage will be in an encrypted form on a University of Dundee password-protected device.

Study assessments will be conducted by the PI and a trained research member of the team under the supervision of the CI.

Data Management System

Data management will be conducted in compliance with TASC SOPs on Data Management, including TASC SOP53 Data Management Systems in Clinical Research. The data management system (DMS) will be EXCEL, as approved by Sponsor. The DMS will be based on the protocol and CRF for the study and individual requirements of the investigators. The CRF will collect only information that is required to meet the aims of the study and to ensure the eligibility and safety of the participant. The study database will be compliant with TASC SOP53 Data Management Systems in Clinical Research. The database is managed in line with all applicable principles of medical confidentiality and data laws. The Data Controller will be the University of Dundee and the Data Custodian will be the CI. The CI may delegate CRF completion but is responsible for completeness, plausibility, and consistency of the CRF. Any queries will be resolved by the CI or delegated member of the study team. Database lock will be conducted in compliance with TASC SOP32 Locking Clinical Study Databases.

Verifying data

Data entry and checking will be undertaken by a single entry with a second look. The data entry and checking process will be decided according to risk. Data that is recorded in the CRF that is not source document in itself will be consistent with the source documents or the discrepancies explained. Checks will be made on all missing values and values out with normal or expected ranges and that values entered

are of the correct type: i.e. numerical instead of text. Logical checks will be performed to ensure consistent reporting between relevant fields and that there are no differences between fields. Data checking will continue until all missing data and/or inconsistent values have been corrected or clarified. When data checking is complete, with no outstanding data queries, the database will be locked, using the Protect Worksheet function of EXCEL, as FINAL RESULTS. The EXCEL spreadsheet will remain archived on the Dundee University secure server for 5 years for the retention of essential documents, thereafter it will be deleted.

STATISTICS AND DATA ANALYSIS

Sample Size Calculation

As this is a PhD project exploring mechanisms of vascular changes, precise power calculations have not been obtained. The results from this PhD project will guide us regarding subsequent power calculations. However, based on our previous work exploring mechanisms of changes in vascular function using various interventions, n=25 subjects will provide at least 80% power and 5% significance to detect a 20% before and after difference within subjects in the vascular function markers proposed in this study.

Proposed Analyses

Changes in blood flow and endothelial function in the lower limbs using laser Doppler perfusion imaging and iontophoresis will be compared primarily within each group and also between patient groups at baseline, after 1 INP session and after the last INP (4 to 8 weeks) is applied. The primary outcome measure will be the change in peak blood flow response to acetylcholine and sodium nitroprusside.

Data will be analysed using widely available statistical analysis software such as SPSS. Analytical methods will include t-tests, chi-square, ANOVA, correlation and regression as appropriate.

STUDY MANAGEMENT AND OVERSIGHT ARRANGMENTS

Study Management

The study will be co-ordinated by a Study Management Group (SMG), consisting of the grant holder Chief Investigator (CI), the Principal Investigator (PI), and the PhD supervisors.

The CI will be responsible for the study data and for resolving any queries. However, the completion of CRFs will be delegated to the members of the research team.

Study procedures are carried out in a dedicated research area with suitably qualified medical, research and nursing staff. Any incidents will be dealt with as necessary at the time, recorded and follow-up care arranged as required.

Inspection of Records

The CI will permit study related monitoring, audits, and REC review. The CI agrees to allow the Sponsor or, representatives of the Sponsor, direct access to all study records and source documentation.

GOOD CLINICAL PRACTICE

Ethical Conduct of the Study

The study will be conducted in accordance with the principles of good clinical practice (GCP). In addition to Sponsorship approval, a favourable ethical opinion will be obtained from the appropriate Research Ethics Committee and NHS Tayside R&D approval before commencement of the study.

Confidentiality and DATA PROTECTION

The CI and trial staff will comply with all applicable medical confidentiality and data protection principles and laws with regard to the collection, storage, processing, and disclosure of personal data. The CI and trial staff will also adhere to the NHS Scotland Code of Practice on Protecting Participant Confidentiality or equivalent. All trial records and personal data will be managed in a manner designed to maintain participant confidentiality. All records, electronic or paper, will be kept in a secure storage area with access limited to appropriate study staff only. Computers used to collate personal data will have limited

access measures via user names and passwords. Personal data concerning health will not be released except as necessary for research purposes including monitoring and auditing by the Sponsor, its designee or regulatory authorities providing that suitable and specific measures to safeguard the rights and interests of participants are in place. The CI and trial staff will not disclose or use for any purpose other than performance of the trial, any personal data, record, or other unpublished, confidential information disclosed by those individuals for the purpose of the trial. Prior written agreement from the Sponsor will be required for the disclosure of any said confidential information to other parties. Access to collated personal data relating to participants will be restricted to the CI and appropriate delegated trial staff. Where personal data requires to be transferred, an appropriate Data Transfer Agreement will be put in place. Published results will not contain any personal data that could allow identification of individual participants.

Insurance and Indemnity

The University of Dundee and Tayside Health Board are Co-Sponsoring the study.

Insurance –The University of Dundee will obtain and hold a policy of Public Liability Insurance for legal liabilities arising from the study.

Tayside Health Board will maintain its membership of the Clinical Negligence and Other Risks Insurance Scheme ("CNORIS") which covers the legal liability of Tayside in relation to the study.

Where the study involves University of Dundee staff undertaking clinical research on NHS patients, such staff will hold honorary contracts with Tayside Health Board which means they will have cover under Tayside's membership of the CNORIS scheme.

Indemnity The Co-Sponsors do not provide study participants with indemnity in relation to participation in the Study but have insurance for legal liability as described above.

ADVERSE EVENTS DEFINITIONS

Adverse Event (AE)	Any untoward medical occurrence in a clinical research participant which does not necessarily have a causal relationship with study participation
Serious Adverse Event (SAE)	A serious adverse event is any untoward medical occurrence that: • results in death • is life threatening • requires hospitalisation or prolongation of existing hospitalisation • results in persistent or significant disability or incapacity • is a congenital anomaly or birth defect • Or is otherwise considered serious

RECORDING AND REPORTING AE

All AEs and/or SAEs will be recorded on the AE Log in the CRF and will be assessed for severity by the clinician from the research team. AEs/SAEs will be recorded from the time a participant consents to join the study until the participant's last study visit. The Investigator will make a clinical judgment as to whether or not an AE is of sufficient severity to require the participant's removal from the study. A participant may also voluntarily withdraw from treatment due to what he or she perceives as an intolerable AE. If either of these occurs, the participant should, if required, be offered an end of study assessment and be given appropriate care under medical supervision until symptoms cease, or the condition becomes stable. AEs/SAEs will be followed up until 30 days after participant's last visit.

The CI or delegate will ask about the occurrence of AEs/SAEs and hospitalisations at every visit during the study. **SAEs which are both unexpected and related to study participation** will be submitted on an HRA NCTIMP Safety Report form to the REC by the CI, within 15 days of becoming aware of the SAE, and copied to the Sponsor Research Governance Office.

Worsening of the condition under study will not be classed as an AE, but will be defined as an outcome. Pre-specified outcome(s) will not be classed as an AE but as an outcome. Elective admissions and hospitalisations

for treatment planned prior to randomisation, where appropriate, will not be considered as an AE. However AEs/SAEs occurring during such hospitalisations will be recorded.

ANNUAL REPORTING REQUIREMENTS

Annual reporting will be conducted in compliance with TASC SOP 15: Preparing and Submitting Progress and Safety Reports in CTIMPs and Non-CTIMPs, as a condition of sponsorship and as a condition of a favourable opinion from a REC. An HRA Annual Progress Report for NCTIMPs will be prepared and submitted by the CI to REC, and copied to the Sponsor, on the anniversary date of the REC favourable opinion.

Any safety reports additional to SAE reports, for example, reports of a DMC, will be sent by the CI to REC, with a Safety Report Form, and to the Sponsor.

STUDY CONDUCT RESPONSIBILITIES

Protocol amendments and breaches

The CI will seek approval for any amendments to the Protocol or other study documents from the Sponsor, REC and NHS R&D Office(s). Amendments to the protocol or other study docs will not be implemented without these approvals.

If this necessitates a subsequent protocol amendment, this will be submitted to the Sponsor for approval and then to the appropriate REC and lead NHS R&D Office for review and approval.

In the event that a breach of GCP or protocol is suspected, this will be reported to the Sponsor Governance Office immediately.

Study Record Retention

Archiving of study documents will be in secure storage for 15 years after end of study.

End of Study

The end of study is defined as last patient last visit (LPLV). The Sponsor and CI have the right at any time to terminate the study for clinical or administrative reasons.

The end of the study will be reported to the Sponsor and REC within 90 days, or 15 days if the study is terminated prematurely. The CI will ensure that any appropriate follow up is arranged for all participants.

A summary report of the study will be provided to the Sponsor and REC within 1 year of the end of the study.

REPORTING, PUBLICATIONS AND NOTIFICATION OF RESULTS

Authorship Policy

The co-applicants will form the core authorship group. Ownership of the data arising from this study resides with the study team and their respective employer. On completion of the study, the study data will be analysed and tabulated, and a clinical study report will be prepared.

Publications

The clinical study report will be used for publication in peer-reviewed scientific journal and presentation (as conference abstracts) at scientific meetings. Investigators have the right to publish orally or in writing the results of the study.

Peer Review

Peer review of the protocol will occur via the resulting publication by the referees of the journal to which the paper (and its protocol) will be submitted.

References:

1. Abdulhannan P, Russell DA, Homer-Vanniasinkam S. Peripheral arterial disease: A literature review. Br Med Bull. 2012;104(1):21–39.

2. Vemulapalli S, Patel MR, Jones WS. Limb Ischemia: Cardiovascular Diagnosis and Management from Head to Toe. Curr Cardiol Rep. 2015;17(7).

3. Vartanian SM, Conte MS. Surgical Intervention for Peripheral Arterial Disease. Circ Res. 2015;116(9):1614–28.

4. Sundby ØH, Høiseth LØ, Mathiesen I, Weedon-Fekjær H, Sundhagen JO, Hisdal J. The acute effects of lower limb intermittent negative pressure on foot macro- and microcirculation in patients with peripheral arterial disease. PLoS One [Internet]. 2017;12(6):e0179001. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28591174%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5462420

5. Sundby ØH, Høiseth LØ, Mathiesen I, Jørgensen JJ, Weedon-Fekjær H, Hisdal J. Application of intermittent negative pressure on the lower extremity and its effect on macro- and microcirculation in the foot of healthy volunteers. Physiol Rep. 2016;4(17):1–11.

6. Rein EB, Filtvedt M, Walløe L, Ræder JC. Hypothermia during laparotomy can be prevented by locally applied warm water and pulsating negative pressure. Br J Anaesth. 2007;98(3):331–6.