DOCTOR OF PHILOSOPHY

Mechanistic Insights into Changes in Blood Flow Following Application of Intermittent Negative Pressure

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Mechanistic Insights into Changes in Blood Flow Following Application of Intermittent Negative Pressure

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A thesis submitted for the degree of Doctor of Philosophy

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Declaration

I hereby declare to be the author of this thesis, the work of which is entirely a record carried out by myself. Unless otherwise stated, all references cited in this dissertation have been consulted. None of the work or material contained within this thesis has been previously submitted and accepted for a higher education degree. Where use has been made of the works of other people, it has been fully referenced or acknowledged accordingly.

Signature of candidate:

Jody McIntosh Dundee, September 2021

Supervisors' Statement

I certify that Jody McIntosh has fulfilled the conditions of ordinance and of the relevant regulations, such that she is qualified to submit this thesis in application for the higher degree of Doctor of Philosophy.

Supervisor Signatures:

Professor Faisel Khan Dundee, September 2021

Mr Stuart Suttie Dundee, September 2021

Summary

Since the 1930's, intermittent negative pressure (INP) has been tested as a means of treating vascular diseases. Recently, the literature has indicated that INP can be used to reduce pain and facilitate wound healing in patients with peripheral arterial disease (PAD) however the physiological mechanism behind these clinical benefits remains unclear. The aim of this study was to evaluate the physiological effects of INP application, in particular vascular function, in both healthy and diseased physiology. Three study groups were assessed: healthy volunteers, PAD patients and volunteers suffering from cold hypersensitivity. INP was applied to participants using the Flow-Ox device with applies a negative pressure of -40mmHg in intermittent cycles of 10 seconds negative pressure followed by 7 seconds of no pressure. Vascular function was evaluated through assessment of blood perfusion and endothelial function using full field laser speckle contrast imaging. Systemic effects of INP on vascular function was tested by measuring arterial stiffness, in particular augmentation index and pulse wave velocity. Blood samples were collected to assess blood inflammatory and oxidative stress markers. In patients suffering from PAD, clinical markers including ankle-brachial pressure index and pain score were also evaluated. This study demonstrated that after one session of INP application, cutaneous blood perfusion and endothelial function local to the site of INP application improves in PAD patients but not in healthy physiology. Further, patients reported a significantly lower pain score after up to 8 weeks of INP application compared to baseline. No other significant findings were recorded for any other the other assessments. As microvascular function is severely impaired in PAD, the potential of using INP as a novel treatment modality capable of improving microvascular circulation and function with benefits in reducing pain and wound healing warrants further investigation.

Chapter 1: Introduction

The purpose of this thesis is to explore the relationship between intermittent negative pressure (INP) and vascular function in both diseased and healthy physiology. This introduction section will overview the scope of this thesis.

The literature documents the use of vacuum systems, negative pressure and, more recently, INP, in vascular medicine as far back as 2000 years ago. Some INP delivery devices have proven to be effective in peripheral arterial disease (PAD) by increasing blood flow to the ischaemic limb resulting in chronic wound healing, pain management and increased walking distance.

PAD is a debilitating atherosclerotic condition in which blood supply to the lower limb is reduced due to stenosis or occlusion in the peripheral arteries resulting in significantly reduced blood supply to the muscles and ischaemia causing pain upon walking, termed intermittent claudication (IC). Critical limb ischaemia (CLI) is characterised by ischaemic muscle pain while at rest with or without associated tissue loss and is ultimately limb and life threatening. There is currently no cure of PAD and current treatment options are limited. First line options involve supervised walking exercise and modification of risk factors such as smoking cessation however compliance to these modifications is extremely poor. Pharmaceutical options including statins and antiplatelet therapy are routinely used to attenuate the atherosclerotic condition as well as manage pain and comorbidities such as diabetes, hypertension, and dyslipidaemia. However, the cost-effectiveness of drug therapy is questioned. In patients suffering from advanced stage CLI, endovascular and open revascularisation aim to increase blood flow to the ischaemic limb and may be recommended to attenuate pain and avoid major limb amputation however a large number of patients are deemed unsuitable for intervention due to age, comorbidities and/or location of stenosis. Intervention comes with risk of significant peri operative morbidity and mortality, and in some instances no such intervention is achievable with the patient faced with amputation or palliation. Amputation can be both mentally and physically hard on patients and requires extensive recovery time and rehabilitation, significant healthcare costs, considerably reduced mobility as well as increased risk of death post-amputation. The care of patients with PAD is challenging and, with an ever-aging population, poses a particular challenge to healthcare resources. A novel treatment modality is required which is non-invasive, has little to no

contraindications, is economical for the healthcare provider and, most importantly, is effective in treating and improving patient quality of life.

Thus far, research surrounding INP has focussed on demonstrating it as a treatment modality for vascular diseases. While the data has provided strong evidence that this is possible, there is little to no literature describing the physiological mechanisms behind the blood flow changes elicited by INP. It is essential the mode of action is better understood to allow the use of INP to be justified and applied correctly. It has been suggested that release of vasodilator substances may occur with INP application however no scientific study had previously evaluated this.

Some of the major vasodilator substances are released by the endothelium, the inner most layer of blood vessels. In healthy physiology, the endothelium plays an essential role in vascular homeostasis with functions including maintenance of vascular tone, immune defence, and angiogenesis. It is well known that atherosclerotic disease, including PAD, begins as a result of oxidative stress which causes endothelial damage. Oxidative stress refers to the imbalance between the production of reactive oxygen species (ROS) and the ability of the antioxidant system to defend against these oxidants. Consequences of endothelial dysfunction include vasoconstriction, platelet aggregation, increased monocyte adhesion and plaque formation. Therefore, the endothelium is routinely investigated in studies to determine endothelial function. Furthermore, oxidative stress elicits an inflammatory response which further exacerbates endothelial dysfunction and contributes to arterial stiffening. Arterial stiffness is a prominent characteristic in patients with arterial disease. Arterial stiffening occurs naturally with age however is accelerated in disease causing the arteries to be less compliant and less able to respond to changes in pressure. Arterial stiffness is often measured to evaluate systemic macrovascular structure as a surrogate marker for vascular damage and has also been shown to be an independent predictor of cardiovascular morbidity and mortality.

As the physiological mechanisms of INP remain unclear, the main objective of this thesis was to investigate the physiological mechanisms behind the positive clinical benefits exhibited with INP application. This thesis presents a study of the effects of INP in three separate groups: healthy volunteers, PAD patients and individuals with cold hypersensitivity. First, a group of healthy volunteers were assessed to determine the effects of INP on healthy

physiology. Furthermore, some healthy volunteers were tested at baseline and after five days of repeated INP application to determine short and long-term effects of INP on healthy physiology. Secondly, PAD patients suffering from CLI with or without tissue loss were recruited and assessed. A small number of CLI patients are not suitable for intervention and are commonly termed 'no option CLI'. These patients tend to be managed with drugs and long-term wound care. It was this group who were the target population in this study as a new intervention may prove to be an effective alternative to either life-long pharmaceutical and wound care management or a life-changing amputation.

Due to the COVID-19 pandemic, recruitment and study activities were halted for nine months from March 2020 to September 2020 and again from January 2021 to March 2021 due to national lockdowns meaning no participant recruitment or assessments could be conducted. This had a significant impact on the planned study groups and procedures. Both a placebo and healthy-age matched control groups to compare against PAD patients were originally planned however due to COVID there was not enough time to complete recruitment and assessment of these groups. As time was limited and patient recruitment was difficult, a new study group was added. A small number of previous studies have evaluated the effects of negative pressure treatment in Raynaud's Phenomenon (RP) with results suggesting negative pressure application can have positive outcomes on digital blood flow.

RP, named after the man who first described it in 1862, describes a cluster of symptoms which arise as a result of vasospasm and reduction in blood flow upon exposure to cold temperature or stress. Symptoms include colour changes, pain and swelling of the digits. Raynaud's phenomenon can be classified as either primary Raynaud's (pRP) or secondary Raynaud's (sRP). Patients who are classified as having sRP experience symptoms due to a secondary, underlying condition such as the connective tissue disorder systemic sclerosis. The pathogenesis of Raynaud's phenomenon is still poorly understood however is thought to involve alterations in the control of vascular tone through endothelial dysfunction and thermoregulation. Management of RP is highly dependent on the severity. Many sufferers with mild symptoms can continue their daily life with small lifestyle modifications including avoiding triggers and smoking cessation. However, for some patients, painful ischaemic attacks are frequent and trophic changes may be present. In these cases, pharmaceutical options are considered including calcium channel blockers. In the most severe cases,

intravenous prostanoid therapy or digital amputation may be considered. Like PAD, treatment options for RP sufferers remain limited and a novel treatment modality may provide an alternative non-invasive option to both pRP and sRP patients.

The additional study group consisted of both healthy volunteers and individuals who reported experiencing cold hypersensitivity in their fingers. A study plan was developed to assess the effects of INP application to the hand on vascular function including endothelial function and arterial stiffness in these two groups as a pilot study for a possible later investigation into the effects of INP on vascular function in RP patients. This study was named the 'Cold Hands study' and will be referred as this throughout this thesis.

This thesis will begin by presenting and reviewing the literature surrounding the topics of the cardiovascular system, vascular disease, and negative pressure. Following on, methodology will present the study design and groups as well as study procedures and data analysis plan. The results will present data collected from all groups and finally the discussion will overview the key findings, discuss what the results may mean and implications of the findings. This thesis will conclude by proposing a physiological mechanism of action of INP based on the previous literature and study results presented here.

Chapter 2: Literature Review of The Cardiovascular System

2.1 The Cardiovascular System and Haemodynamics

2.1.1 The Cardiovascular System

The human cardiovascular (CV) system is a closed organ circulatory system comprised of the central heart muscle and blood vessels which populate the periphery. It is the job of the circulatory system to deliver blood, which carries oxygen, nutrients and hormones, to organs of the body and to remove waste products produced by cellular metabolism. The heart itself is a muscle, composed of myocytes. On average, the heart beats 70 times per minute. Problems with the heart muscle can cause changes to heart rate, blood pressure and lead to events such as cardiac arrest. There are two compartments to the CV system: the pulmonary and systemic circuits. The pulmonary circuit runs from the right ventricle, through the lungs where blood is oxygenated, and then travels back to the left atrium to be distributed throughout the peripheral system. The systemic circuit pumps oxygenated blood from the left ventricle to the peripheral tissues and organs including the brain which then travels back to the heart, arriving at the right atrium.

Cardiac output (CO) refers the amount of blood which the heart pumps in one minute and is equal to heart rate multiplied by stroke volume (SV) which is the volume of blood ejected from the ventricles with each cardiac contraction. CO is dependent on both preload and afterload as well as heart rate. Preload refers to the stretching of cardiac muscle cells before cardiac contraction with the greater the stretch of muscle cells meaning greater force for contraction. When preload increases, cardiac output also increases. Afterload refers to the force which the heart, in particular the ventricles, must contract for blood to be ejected. When systemic vascular resistance decreases, upon vasodilation of the peripheral vessels, afterload decreases and when vascular resistance increases, afterload increases. When afterload decreases, stroke volume increases thus CO increases. Exercise is known to decrease preload and afterload and therefore increase CO.

There are five different varieties of blood vessel within the human body: arteries, arterioles, capillaries, venules and veins, each varying in size. Veins and arteries, the macrovessels, range from 30mm – 100um. Arterioles and venules are typically <100um in size while capillaries, the smallest microvessels, are 3um-10um. Arteries and arterioles carry blood

away from the heart to tissues and organs. Veins and venules carry blood, containing waste, back to the heart. Capillaries are a single layer of endothelial cells which allows for close contact with tissues and rapid diffusion of oxygen, nutrients and waste to and from the tissue. The vessels can be divided into the macrocirculation and microcirculation. Macrocirculation consists of the large vessel arteries and veins which carry blood over large distances to the target organs and offer little resistance the flow. The microcirculation is made up of the arterioles, venules and capillaries and is responsible for delivery and diffusion of blood borne substances to the tissue. The microcirculation is responsible for oxygen and nutrient delivery in the tissues. Virtually all cells in the human body are in close proximity to a capillary bed to allow for rapid diffusion of oxygen and nutrients for aerobic metabolism to take place.

The wall of arteries and veins are thick and consist of three layers, tunica intima, tunica media and tunica adventitia (Figure 2.1). The tunica intima is the inner most layer composed of endothelial cells. This layer is discussed in depth in section 2.2. The tunica media is the thick middle layer of arteries and is thinner in veins. This layer's major component is vascular smooth muscle cells but also contains elastin fibres and connective tissue. The tunica adventitia/externa composes the outer layer of vessels and makes up the thickest layer of arteries. It is composed of connective tissue, lymphatic vessels, capillaries and nerve fibres which innervate other layers. The arterial wall is thicker than the wall of veins due to the high pressure exerted on the arteries from ejected blood coming from the heart. Veins, but not arteries, have one-way valves which aid in blood return to the heart and prevents blood from flowing backwards.

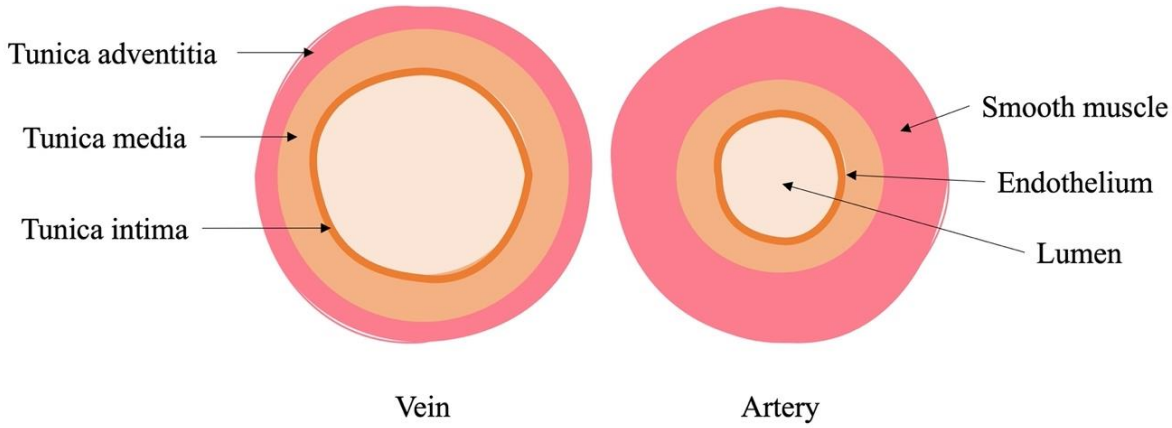

Figure 2.1 ***Cross section of vein and artery.*** Veins and arteries are composed of three layers, the tunica adventitia, tunica media and tunica intima. The lumen is the middle part of the vessel through which blood flows. The tunica adventitia, the outmost layer, is composed of connective tissue, lymphatic vessels, and nerve fibres. This layer is thicker in arteries in veins due to the high pressure of blood flow. The tunica media is the middle layer and compared of vascular smooth muscle cells. The tunica intima is composed of endothelial cells, making up the endothelium which encounters the flowing blood.

2.1.2 Haemodynamics – Flow, Pressure and Resistance in the Cardiovascular System

Haemodynamics refers to the physical properties that govern how blood physically flows. Blood flows throughout the body due to pressure differences between proximal and distal parts of the vessels. The heart produces a pressure pulse upon systolic contraction which travels throughout the periphery and generates the pressure gradient required for blood flow. As the blood vessels get smaller, resistance to flow increases therefore, in the periphery, the pressure falls due to resistance. When the heart relaxes during diastole, arterial elastic recoil forces blood out of the arteries, into veins and back to the heart.

Applying Ohm's law, which states that current (I) is equal to the difference in voltage between two points (ΔV) divided by the resistance (R), to haemodynamics tells us that the rate of blood flow (Q) is dependent on the pressure gradient between arteries and veins (ΔP) and resistance to flow (R).

$$Q = \frac{\Delta P}{R}$$

According to Hagen-Poiseuille's equation, resistance is determined by three factors: vessel radius (r), length (L) and blood viscosity (η) (Pfitzner 1979). The rate of blood flow (Q) is proportional to the pressure gradient across the vessel wall (ΔP) and the vessel radius meanwhile Q is inversely proportional to the vessel length and blood viscosity. According to this, an increase in radius reduces the resistance, a slight change in the vessel radius will bring about a large change in resistance to blood flow. For example, if the blood vessel diameter were to double, resistance would decrease, and rate of flow increase 16-fold.

$$Q = \frac{\Delta P \ \pi \ r^4}{8 \ \eta \ L}$$

The vessel length and viscosity do not normally change significantly in healthy physiology however vessel radius is dynamic and frequently changes to allow for alterations in blood pressure and flow in response to haemodynamic challenges such as exercise. Vessel radius is regulated by the fine balance between vasoconstriction and vasodilation with only small changes to the radius needed to bring about large changes in flow. However, it should be noted that this equation has several assumptions; (a) laminar flow conditions, (b) the fluid is Newtonian and (c) vessels are nonbranching, ridged and straight. In healthy physiology, flow does not conform exactly to this theory. It is true that blood is not a Newtonian fluid and blood flow is not steady. Furthermore, blood vessels are not rigid tubes but in fact highly elastic, branching vessels with bifurcate. Therefore, while the equation is a good explanation of the influences of pressure and resistance on blood flow and demonstrates the influence of radius on resistance and flow, should not be taken literally.

2.1.3 Transmural Pressure

Transmural pressure is the pressure difference across the blood vessel wall. A rise in transmural pressure i.e. a greater pressure difference across the blood vessel wall, will increase blood vessel diameter through vessel dilation (Rhoades & Bell 2012).

Arterial compliance is an essential component of blood vessels and dictates how well vessels can expand and contract passively in response to continuous changes in pressure. Compliance

(C) describes the relationship between changes in blood volume (V) with varying pressure (P).

$$C = \frac{\Delta V}{\Delta P}$$

Compliance controls vessel radius and therefore resistance to flow. Veins are 20 times more compliant than arteries meaning these vessels allow for large increases in blood volume with little increase in blood pressure therefore veins are able to accommodate higher volumes of blood with only small alterations in pressure than arteries, in which high volumes elicit significant increases in pressure. With vasoconstriction, the compliance of a vessel decreases. Arterial stiffening, which occurs naturally with age but also with disease, also reduces arterial compliance. While an increase in transmural pressure dilates the blood vessel, and compliance allows the vessel to dilate, elastic recoil, an inward force exerted on the blood vessel wall, prevents the vessel from bursting. When transmural pressure is higher than recoil pressure, the vessel will expand.

2.2 The Endothelium

2.2.1 Structure and Function of the Endothelium

The endothelium composes the inner most layer of the blood vessel wall and comes into contact with flowing blood (Figure 2.2). The endothelium is composed of a single layer of squamous cells known as endothelial cells. Endothelial cells line all blood vessels of the arterial system including arteries, veins and capillaries. Endothelial cells align themselves along the direction of blood flow, forming an interface between the underlying smooth muscle and vessel lumen allowing interaction with circulating blood molecules.

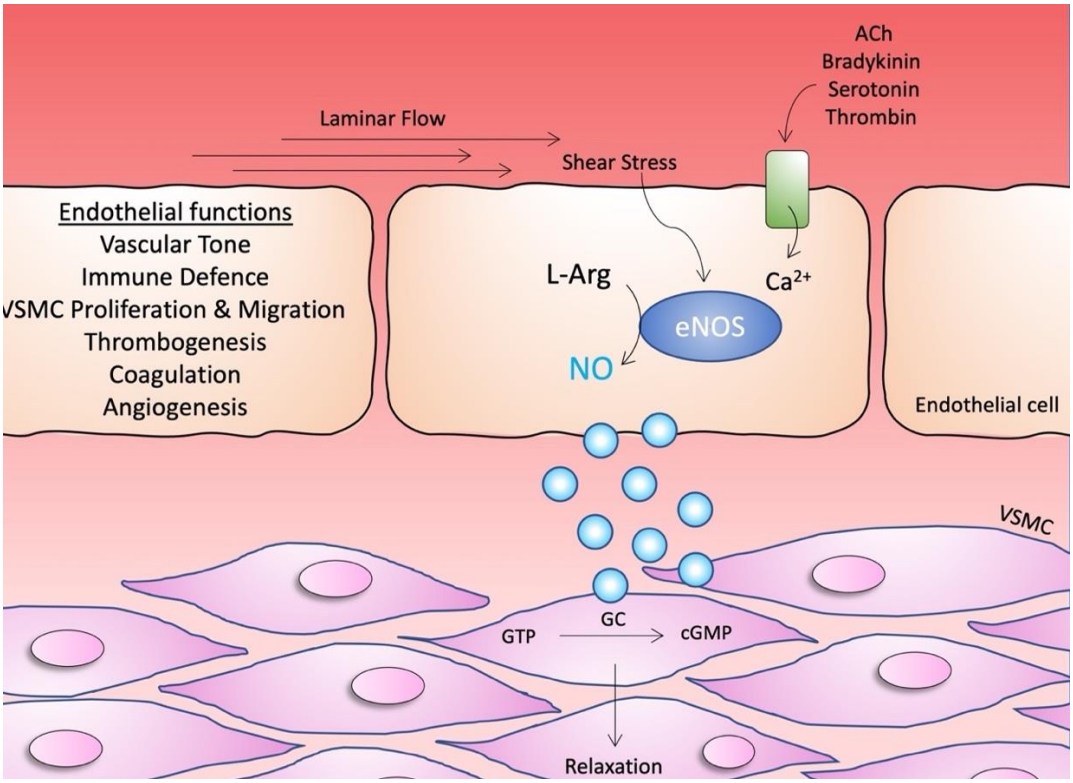

Figure 2.2 ***The Endothelium.*** The endothelium is composed of endothelial cells and serves many functions including controlling vascular tone, immune defence, VSMC proliferation and migration, thrombogenesis, coagulation and angiogenesis. Endothelial cells produce NO, a potent vasodilator, which can be elicited through several mechanisms including shear stress and vasoactive agonist receptor binding such as ACh, bradykinin, serotonin and thrombin. Endothelial NOS converts L-arginine to NO. NO diffuses from the endothelial cell to underlying vascular smooth muscle where it stimulates GC to convert GTP to cGMP and elicit muscle relaxation thus promoting dilation of the blood vessel. ACh = acetylcholine, VSMC = vascular smooth muscle cell, L-arg = l-arginine, NO = nitric oxide, NOS = nitric oxide synthase, GTP = guanosine triphosphate, GC = guanylyl cyclase, cGMP = cyclic guanosine monophosphate.

Originally, the endothelium was thought to only serve its function as a barrier between the vascular smooth muscle and lumen. Further research uncovered several functions of the endothelium, revealing it as an essential component for maintaining vascular health. In the 1980s, Furchgott and Zawadzki conducted a historical experiment revealing the endothelium as an essential component in the blood vessel wall for contraction and expansion (Furchgott and Zawadzki 1980). The endothelium is often said to be the master regulator of vascular homeostasis as it serves roles in inflammation, apoptosis, leukocyte trafficking, vascular tone, immune defence, smooth muscle cell proliferation and migration, thrombogenesis, fibrinolysis, coagulation and angiogenesis.

The endothelium responds to various external stimuli including hormones, neural input and haemodynamic forces. Endothelial cells are activated upon exposure to shear stress or vasoactive agonists including acetylcholine (ACh), histamine, noradrenaline, substance P, isoproterenol, bradykinin, thrombin and serotonin. Some of these agonists (ACh, serotonin, noradrenaline and histamine) promote vasoconstriction in the absence or dysfunction of the endothelium. In healthy vessels, ACh promotes endothelial-dependent vasodilation whereas in disease, such as atherosclerosis where the endothelium is damaged, ACh promotes vasoconstriction through direct interaction with the smooth muscle. This has been demonstrated using experimental models. When the endothelium is removed, addition of ACh results in smooth muscle cell constriction (Ludmer et al 1986).

Angiogenesis is the formation of new blood vessels originating from existing ones to create collateral flow and occurs in response to damage or growth. Hypoxia and ischaemia stimulate growth factor production, through the transcription factor hypoxia-inducible factor-1 (HIF-1), which promotes angiogenesis. The response is mediated by nitric oxide (NO) and is triggered in response to shear stress and growth factors, namely vascular growth factor (VEGF). VEGF is a proangiogenic growth factor, and its actions depend on the stimulation of endothelial receptors. Levels of angiogenesis can be assessed through measurement of VEGF-A levels. In eNOS knock out (KO) hind limb ischaemic mice, angiogenesis is impaired however intramural injection of eNOS can improve blood flow in these mice (Ziche et al 1997, deMuinck et al 2005). In atherosclerosis, the body responds by promoting angiogenesis to allow for development of collateral circulation in the ischaemic tissue. Compared with healthy individuals, PAD patients have higher levels of VEGF-A and the level increases as limb ischaemia/disease severity worsens suggesting heightened levels of angiogenesis (Wieczor er al 2019). However, angiogenesis alone is not sufficient to counteract the haemodynamic imbalance/blockage caused by an atherosclerotic lesion and full restoration of flow cannot be achieved by angiogenesis in atherosclerosis.

The endothelium serves as a barrier, between the underlying smooth muscle and vessel lumen, allowing for selective permeability of molecules by means of intercellular junctional complexes and transcellular vesicles. Endothelial permeability can be altered by haemodynamic forces, inflammation and oxidative stress. Increased permeability of the

endothelium can be atherogenic due to increased leukocyte infiltration leading to plaque formation. HDL-C and exercise can help to maintain endothelial barrier integrity.

One of the endothelium's most prominent roles is to control vascular tone by maintaining a fine balance between vasodilation and vasoconstriction. To control vascular tone, the endothelium releases numerous vasoactive substances including the vasodilators NO, prostacyclin (PGI2) and endothelium derived hyperpolarising factor (EDHF). Endothelium derived vasoconstrictors include endothelin-1 (ET-1) and vasoconstrictor prostaglandins.
NO is a potent vasodilator which governs basal vascular tone. NO is produced inside the endothelial cell in response to shear stress and vasodilator agonists. Endothelial nitric oxide synthase (eNOS) catalyses the conversion of the substrate L-arginine into NO, with L-citrulline being a by-product. eNOS is a calmodulin-dependent enzyme with binding sites for four cofactors: nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and tetrahydrobiopterin (BH_4) as well as another binding site for the substrate L-arginine. eNOS is located in caveolae (in the cell membrane) and is inhibited by caveolin-1 binding to calmodulin. Calcium binding to calmodulin releases the inhibition of calveolin-1, activating eNOS (Greif et al 2003). eNOS expression can be increased by shear stress by means of Ca2+ signalling. NO production consists of two phases; rapid and sustained. The rapid phase is dependent on Ca2+ intake via TRP channels on the endothelial surface while the sustained phase is Ca2+-independent but flow dependent. Sustained NO formation requires phosphorylation of eNOS by Akt to PKA (Wang et al 2016) (Kuchan & Frangos 1994a, Kuchan & Frangos 1994b). L-arginine analogues N(G)-monomethyl L-arginine (L-NMMA) and N(G)-nitro L-arginine methyl ester (L-NAME) are pharmacological inhibitors of eNOS. eNOS synthesis can be inhibited by isoprenoid geranylgeranyl pyrophosphate which is part of the cholesterol synthesis pathway. Cofactor BH4 is impaired by oxidative stress which can lead to uncoupled eNOS. Uncoupled eNOS produces ROS, instead of NO, and therefore can promote a prooxidant environment. Another form of NOS, inducible NOS (iNOS), is induced during an immune response and stimulated by cytokines. iNOS is also capable of producing NO.

NO mediates endothelium-dependent vasodilation and works by opposing the effects of endothelium-derived vasoconstrictors. NO inhibits platelet aggregation, leukocyte adhesion and infiltration, oxidation of LDL, and smooth muscle cell proliferation and thus promotes

an anti-atherogenic environment. In the presence of oxygen, NO has a half-life of 3-5seconds and is rapidly metabolised into nitrite and nitrate. NO half-life can be prolonged by free radical scavengers such as SOD. NO can be pharmacologically inhibited by asymmetric dimethylarginine (ADMA).

NO exerts its effects through stimulation of guanylyl cyclase (GC) in a number of blood and vascular wall components including platelets, red blood cells, leukocytes and smooth muscle cells. In platelets, GC activation leads to inhibition of platelet activation, adhesion and aggregation. In RBCs, NO enhances oxygen delivery through reaction with haemoglobin. In leukocytes, GC activation reduces adhesion. In smooth muscle cells, NO stimulates GC to convert guanosine triphosphate (GTP) to the secondary messenger cyclic guanosine monophosphate (cGMP) leading to decreased intracellular Ca2+ and muscle cell relaxation. As well as GC activation, the actions of NO can also be exerted by s-nitrosylation. S-nitrosylation is a reversible post-translational modification which alters the function of target proteins including those involved in cell proliferation, apoptosis, exocytosis and blood flow.

Although NO is the major contributor to endothelial-dependent vasodilation, it does not account for all endothelium-dependent vasodilation. Prostacyclin (PGI2) is another effective vasodilator produced in and released by the endothelium. Cyclooxygenase (COX) enzymes catalyse the production of PGI2 from prostaglandin H2 (PGH2). PGI2 does not contribute to maintenance of basal vascular tone but has been shown to play a compensatory role in individuals with low NO bioavailability (Szerafin et al 2006). Prostacyclin works alongside NO to inhibit platelet aggregation, reduce leukocyte adhesion and reduce SMC proliferation.

When NO and prostacyclin are completely inhibited, a vasodilator response may still be present due to EDHF. EDHF promotes VSMC relaxation through hyperpolarisation by means of increasing K^+ conductance. The exact definition of what EDHF exactly is has not been fully elucidated. It has been proposed that hydrogen peroxide (H_2O_2) is an EDHF. Endothelial cells produce H_2O_2 from sources including eNOS, cyclooxygenases (COX) and NADPH oxidases found inside cells. Animal studies have demonstrated that as blood vessel size decreases, the contribution of EDHF to vascular tone increases and furthermore EDHF-mediated vasodilation is attenuated in pathogenic conditions such as diabetes mellitus (Shimokawa et al 1996, Fukao et al 1996). EDHF may be more prominent than NO in the

small vessels including arterioles and therefore EDHF plays a significant role in control of vascular resistance and blood flow. When NO and PGI2 are suppressed, under some pathogenic conditions, EDHF can be upregulated to allow for maintenance of physiological blood flow.

Vasoconstrictors endothelin-1 (ET-1) and prostaglandins are produced and released by the endothelium. ET-1 is the most potent endogenous vasoconstrictor discovered. It exerts its effects by opposing the effects of vasodilators, acting on the smooth muscle cells to promote constriction. However, the dominant physiological pathway favours vasodilation. Angiotensin II (AngII) is part of the renin-angiotensin system and regulates blood pressure. AngII can promote ET-1 production by endothelial cells and stimulate smooth muscle cell proliferation.

Vascular tone is also modulated by sympathetic innervation in the blood vessel. Sympathetic nerves innervate the arterial wall and release noradrenaline which promotes vasoconstriction of VSMC. Substances released by the endothelium modulate the effects of noradrenaline. NO can inhibit noradrenaline release and thereby attenuate vasoconstriction. When the endothelium is damaged, less vasodilators are produced and/or released from the endothelium and the adrenergic vasoconstrictor tone is unopposed.

The vascular system is consistently exposed to two forces: fluid shear stress and cyclic strain. Shear stress and cyclic strain are haemodynamic forces exerted on the endothelium generated by pulsatile blood flow, both of which have been identified as modulators of vascular function through their interaction with endothelial cells and vascular smooth muscle cells (VSMCs). Stretch elicits intracellular signalling and changes in gene expression within endothelial and VSMCs allowing for communication between the two cell types and thereby control of vascular function. For example, shear stress can promote nuclear translocation of nuclear factor erythroid 2-related factor (Nrf2) and thereby promote antioxidant gene expression (Hsieh et al 2009). Shear stress is a parallel frictional force exerted on the endothelium during normal blood flow. Cyclic strain is a perpendicular force acting on the vascular wall due to blood pressure. Endothelial cells response to flow. Upon flow sensing, there is ion influx (K^+ and Ca^{2+}) leading to NO production. Sustained flow elicits activation of MAPK, eNOS and Akt leading to sustained NO production (the two phases of NO

production; rapid and sustained) (Boo et al 2002). Shear stress responses increase as the shear stress magnitude increases (Yamamoto et al 2000). Disturbances in these forces can elicit changes in normal endothelial functioning. For example, abnormal stretch and flow patterns can occur in chronic hypertension which leads to arterial wall thickening and a reduced vasodilator response (Lu & Kassab 2011).

Blood flow is mostly laminar with unidirectional flow observed in most vessels of the circulatory system. However, in areas of high resistance, such as bifurcation points, blood flow can be disturbed and oscillatory. These areas are exposed to low shear stress, altering the signals sent to the endothelium. Vessel regions which are exposed to unidirectional, laminar flow exhibit endothelial cell elongation and alignment in the direction of blood flow. Furthermore, under these conditions, there is low cell proliferation and atheroprotective gene expression in endothelial cells. In areas exposed to disturbed flow, endothelial cells are not orientated in a particular pattern and are rounded in shape. These areas exhibit high endothelial cell turnover, increased adhesion molecule expression and are prone to development of atherosclerotic lesions.

In order to sense these varying haemodynamic forces and flow patterns, endothelial cells express a number of mechanosensors and mechanotransducers which sense the forces and elicit endothelial intracellular signalling cascades to bring about a response. Mechanotransduction is mediated by numerous mechanosensors. Mechanosensors sense flow force and changes in force meanwhile mechanotransducers are required for conductance of signals generated by the force. The mechanisms underlying mechanosensing and transduction remain somewhat unclear. To date, at least ten endothelial mechanosensors have been identified including glycocalyx, ion channels, tyrosine kinase receptors, G-protein coupled receptors, primary cilia, integrins and cell adhesion molecules found in varying locations of the endothelial cell: luminal, junctional, apical and basal. An impairment in the endothelial mechanosensing and mechanotransduction pathway can lead to atherogenesis (Ranade et al 2014). Experiments with mechanosensor-deficient animals have shown increased atherosclerotic development compared to those expressing mechanosensors (Cahill et al 2016).

Mechanosensors present on the endothelial surface include pressure-sensitive ion channels with one such example being PIEZO. PIEZO was first discovered as a novel ion channel family in 2010 by Coste (Coste et al 2010). PIEZO is a non-selective cationic, pressure-sensitive ion channel which is expressed by endothelial cells and plays a key role in vascular development, blood pressure control and mechanotransduction. Shear stress elicits PIEZO1 activation leading to Ca^{2+} influx, membrane depolarisation, activation of Ca^{2+}-dependent signalling pathways and phosphorylation of Akt (also known as protein kinase B), eNOS activation, ending in NO production and release (Figure 2.3). PIEZO activation aids in endothelial cell alignment through remodelling of the extracellular matrix. Loss of PIEZO expression can lead to high blood pressure due to loss of mechanosensing (Zhong et al 2018). Other ion channels said to be mechanosensors are Kir2.1 and TRPV4. TRPV4 is activated by stimuli including mechanical flow forces. Stimulation of TRPV4 leads to hyperpolarisation of VSMCs and vasodilation. TRPV4 channels, like other mechanosensors, are involved in regulation of vascular tone and blood pressure. TREK1 is also present in the vascular system and deletion from the microcirculation leads to reduced vasodilator response to ACh (Garry et al 2007).

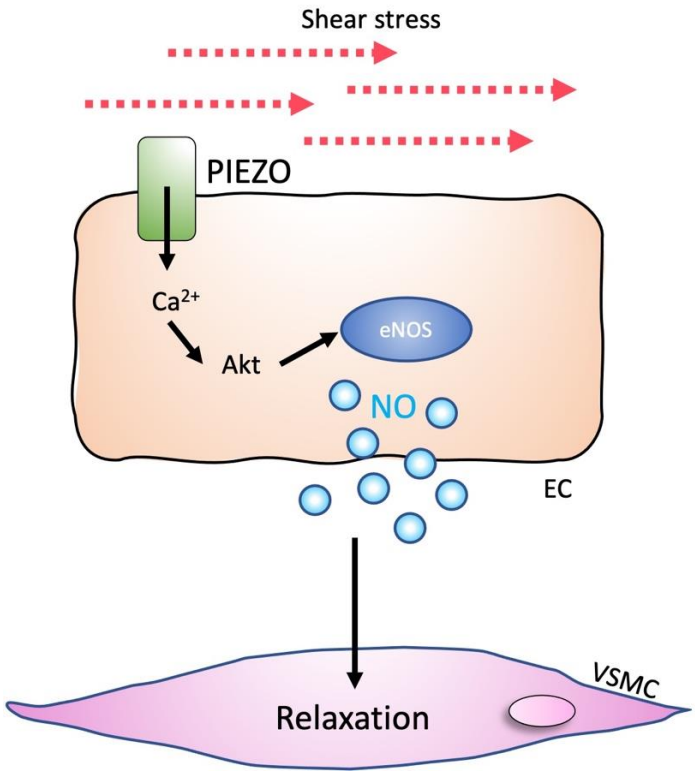

Figure 2.3 ***PIEZO as a mechanosensor.*** PIEZO, an ion channel, is located in the endothelial cell membrane and senses shear stress. In response to shear stress, Ca^{2+} enters the endothelial cell through PIEZO which elicits intracellular Ca^{2+}-dependant signalling and Akt activation of eNOS which produces NO. NO diffuses to the underlying smooth muscle where it elicits relaxation and vessel dilation. VSMC = vascular smooth muscle cell, EC = endothelial cell, NO.= nitric oxide, eNOS = endothelial nitric oxide synthase.

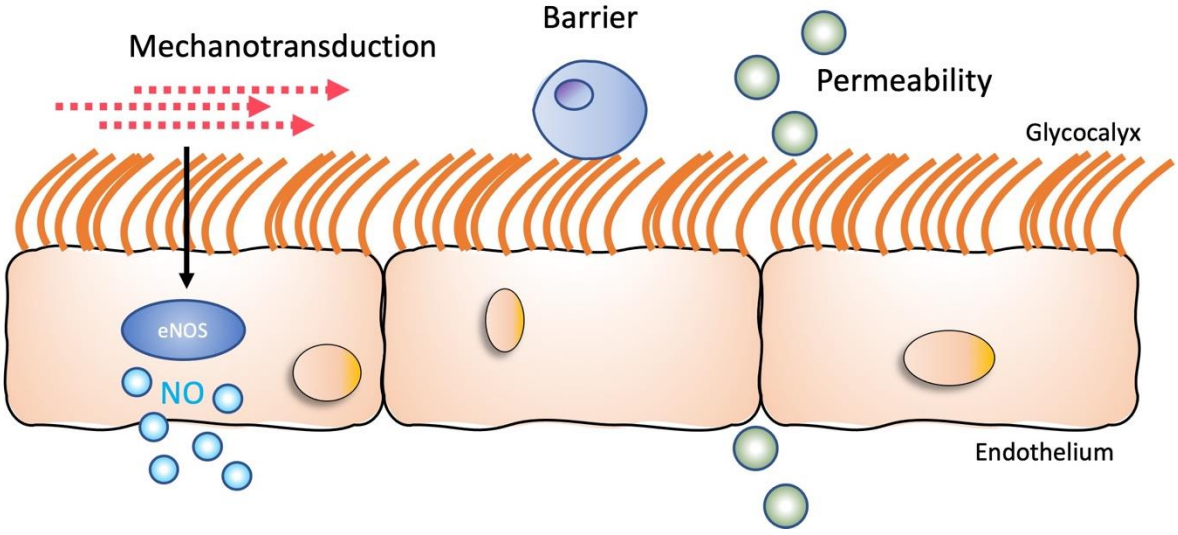

Figure 2.4 ***Endothelial Glycocalyx.*** Endothelial glycocalyx are present on the endothelial luminal surface where they sense shear stress. Upon sensing of mechanical force, intracellular signalling is elicited which results in eNOS activation and NO production. The glycocalyx also serves roles in barrier function and maintenance of endothelial permeability. NO = nitric oxide. eNOS = endothelial nitric oxide synthase.

Endothelial glycocalyx form a thin layer (0.5um) on the luminal endothelial surface where they sense mechanical stretch and signal to the endothelial cell to produce NO (Figure 2.4). The glycocalyx layer is composed of proteoglycans, glycoproteins and glycosaminoglycans (GAGs). GAGs consist of heparan sulfate (HS), hyaluronic acid (HA) and chondroitin sulfate (CS). As well as being a mechanosensor, glycocalyx serves as a selective permeability barrier with anti-inflammatory and anti-adhesive properties. The glycocalyx holds essential enzymes and signalling molecules required by the endothelium. Furthermore, the glycocalyx is said to attenuate the forces acting on the endothelial surface (Givens et al 2016). Glycocalyx also play a role in angiogenesis by modulating endothelial cell migration, proliferation, and differentiation through interactions with growth factors (VEGF, FGF) and extracellular matrix components. Upon shear stress, there is reorganisation of the glycocalyx components which brings about tension in the lipid bilayer and activation of the mechanotransduction machinery. These signals are propagated throughout the endothelial cell through cytoskeletal connections eliciting a variety of mechanically sensitive ion channels leading to Ca^{2+} inflow, activation of Ca^{2+} signalling, eNOS activation, NO production and VSMC relaxation. Glycocalyx adapt and remodel in response to continuous shear stress. The endothelial glycocalyx has been shown to be essential for the endothelial response to shear stress. Enzymatic removal of HS inhibits shear stress elicited NO production however this had no effect on PGI2 production suggesting PGI2 production may be mediated by another mechanosensor (Thi et al 2004, Pahakis et al 2007). Furthermore, enzymatic removal of glycocalyx components, namely HS and HA, can lead to loss of endothelial cell alignment and increased inflammation (Nauli et al 2008, Thi et al 2004, Van der Heiden et al 2008, Weinbaum et al 2007). The glycocalyx can be visualised and measured through several direct and indirect methods such as transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM) and intravital microscopy (IM) (Kang et al 2017).

2.2.2 Endothelial Dysfunction

The endothelium plays a significant role in the normal physiological regulation of blood vessels but, when dysregulated, also pays an important role in the pathogenesis of disease (Figure 2.5). The endothelium can become damaged upon chronic exposure to risk factors including hyperglycaemia and dyslipidaemia. Endothelial damage has many structural and functional consequences. Endothelial dysfunction results in irreversible changes to the

physiological functioning of endothelial cells and alters the balance between vasodilation and vasoconstriction as well as increasing endothelial permeability, leukocyte adhesion, LDL oxidation, smooth muscle cell proliferation and migration, platelet aggregation and cytokine production. Together, these all contribute to the formation of atherosclerosis. Ludmer was first to demonstrate impaired endothelial function in humans with atherosclerosis (Ludmer et al 1986). It is now well known that endothelial dysfunction is present in the pre-clinical stages of atherosclerosis.

The most distinctive aspect of endothelial dysfunction is impaired endothelium-dependent vasodilation, mediated by NO, and increased bioavailability of vasoconstrictor ET-1. A decrease in NO bioavailability may occur for several reasons: reduced eNOS expression, eNOS uncoupling or elevated NO degradation by reactive oxygen species (ROS). Reduced NO bioavailability results in atherogenic events: platelet aggregation, leukocyte adhesion and infiltration, smooth muscle cell proliferation, vasoconstriction, inflammation and oxidative stress. Furthermore, endothelial dysfunction disturbs the endothelial anticoagulant, fibrinolytic, anti-thrombotic state leading to a change in blood fluidity which can result in haemorrhage or clot formation.

Miralles et al investigated endothelial function in patients with PAD. 82 PAD patients (50 IC and 32 CLI) were recruited, and endothelial function assessed by flow-mediated dilation (FMD) and compared against 41 healthy controls. FMD was lower in patients than healthy however there were no significant differences in FMD value between the two patient groups. This provides strong evidence that endothelial dysfunction is present in PAD but also suggests dysfunction does not increase with disease severity and supports the hypothesis that endothelial dysfunction is an event which takes place during the very early stages of disease (Miralles et al 2008). Poredos et al investigated differences in micro- and macro-vascular endothelial function between PAD patients and healthy controls using peripheral arterial tonometry (PAT) and flow-mediated dilation (FMD). Overall, PAD patients had reduced FMD, NMD and PAT compared to controls suggesting micro- and macro-circulation endothelial dysfunction (Poredos et al 2016) (Komai et al, 2008). Reduced FMD (endothelium-dependent dilatation) and NMD (endothelium-independent dilatation) in PAD patients suggests the disease effects more blood vessel layers than the endothelium alone. In a five-month prospective follow-up study, PAD patient endothelial dysfunction was found to

be correlated with cardiovascular (CV) risk as patients who recorded the lowest FMD had the highest risk of CV event including cardiac, cerebrovascular and peripheral events (Brevetti et al 2003).

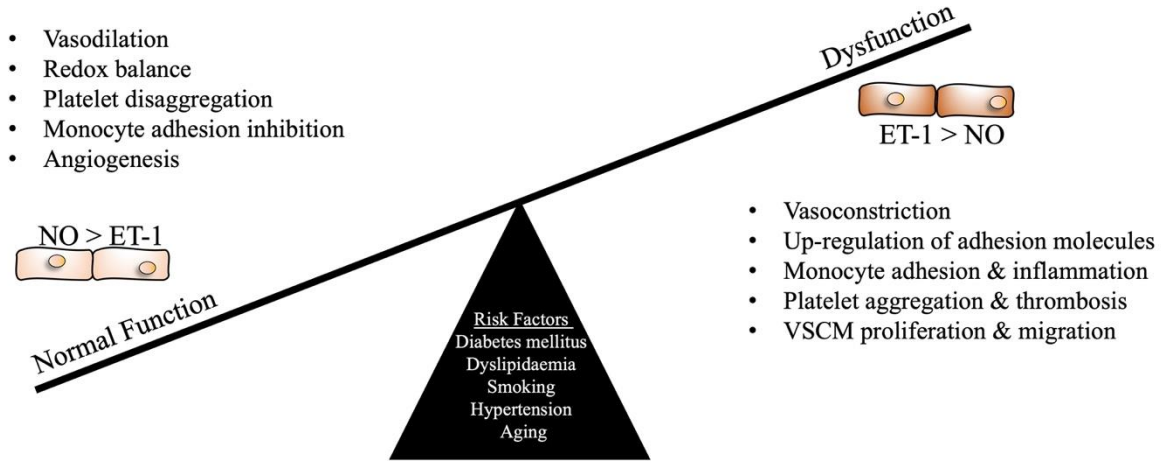

Figure 2.5 ***Endothelial dysfunction.*** Under normal, healthy physiological conditions, the endothelium promotes vasodilation, redox balance, platelet disaggregation, monocyte adhesion inhibition and angiogenesis. Under these conditions, NO levels are greater than ET-1, a potent vasoconstrictor, levels. Chronic exposure to cardiovascular risk factors including diabetes mellitus, dyslipidaemia, smoking, hypertension and aging results in endothelial dysfunction. A dysfunctional endothelium promotes vasoconstriction of the blood vessel, upregulation of adhesion molecules which promotes monocyte adhesion and an inflammatory environment. Furthermore, endothelial dysfunction results in platelet aggregation, thrombosis, VSMC proliferation and migration. In this state, ET-1 overtakes NO as the main controller of vascular tone. VSMC = vascular smooth muscle cell, NO = nitric oxide, ET-1 = endothelin-1.

Development of atherosclerosis is a slow process and occurs over many years due to chronic exposure to cardiovascular risk factors with symptoms normally ensuing later in life. Atherosclerotic risk factors associated with endothelial dysfunction include smoking, diabetes, sex hormone imbalances (menopause), aging, hypertension and hypercholesterolaemia (Davignon and Ganz 2004). Risk factors are known to alter haemodynamic forces including shear stress and therefore alter endothelial function. The process of atherogenesis begins with exposure to these risk factors eliciting oxidative stress and an inflammatory response. In response to inflammatory stimuli, endothelial cells express adhesion molecules leading to immune cell filtration and plaque formation (Figure 2.6).

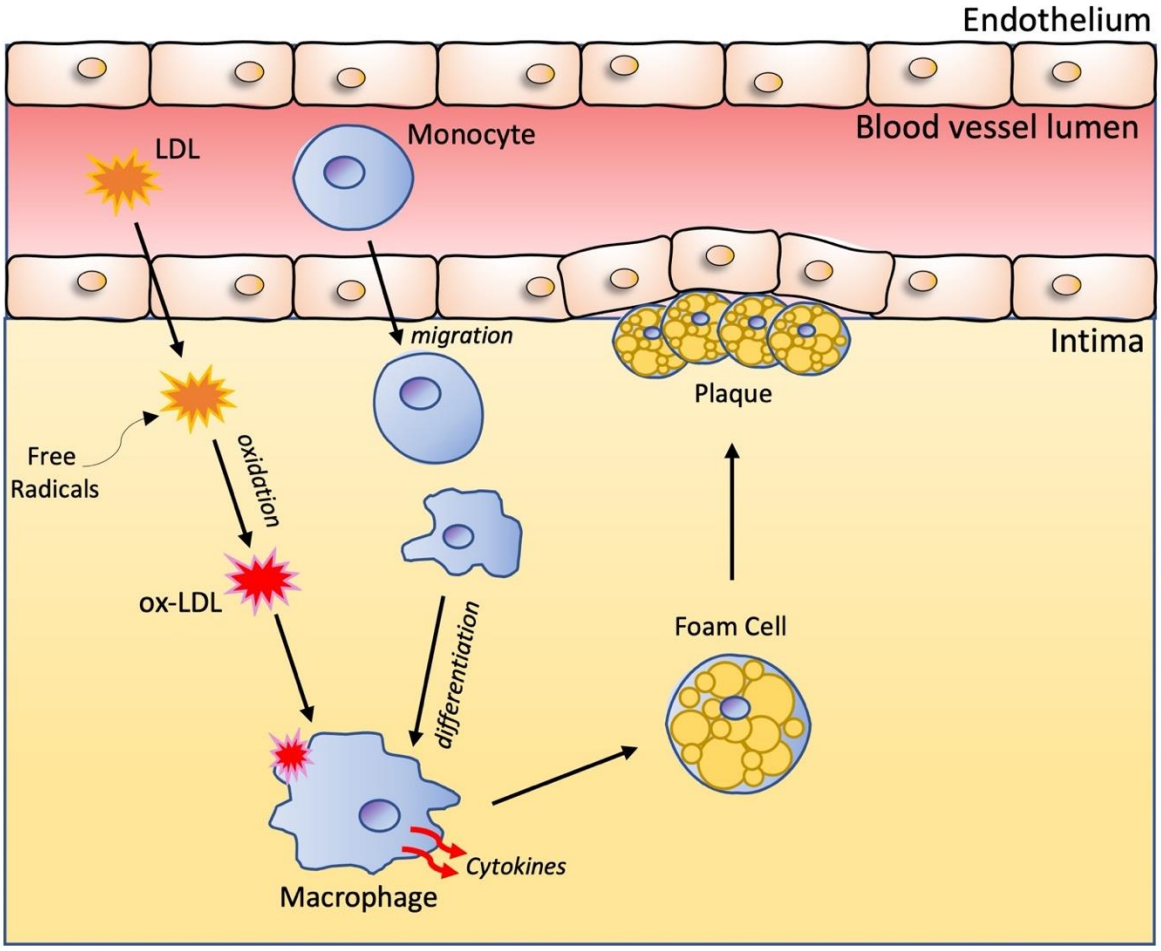

Figure 2.6 ***Process of atherosclerosis development.*** When the endothelium is exposed to cardiovascular risk factors and becomes dysfunctional, atherosclerosis development occurs. LDL and monocytes filtrate through the endothelial barrier as its integrity is compromised. LDL are oxidised by free radicals to ox-LDL. Monocytes differentiate into macrophages which are activated by ox-LDL to release inflammatory cytokines and form foam cells. Foam cells aggregate and form the atherosclerotic plaque which, which large enough, will impede blood flow. LDL = low-density lipoprotein, ox-LDL = oxidised low-density lipoprotein.

In atherosclerotic vessels, endothelial dysfunction leads to favouring of a vasoconstrictor, prothrombotic environment. The stenosis has numerous haemodynamic effects: turbulent flow, abnormal shear stress patterns, decreased systolic blood pressure distal to the stenosis and therefore decreased ankle-brachial pressure index (ABPI), a marker of arterial disease. In individuals with atherosclerotic disease, there is an inability to increase blood flow in response to demand during situations of exercise leading to demand/perfusion mismatch and tissue ischaemia (Campia et al 2019).

Although the basic mechanisms of endothelial dysfunction and consequential atherogenesis are mostly well understood and widely accepted, as previously described, the exact molecular events leading to endothelial dysfunction are less well understood. Kruppel-like factor 2 (KLF2) is a transcription factor said to be involved in endothelial function and in particular, barrier function. KLF2 is downregulated by disturbed blood flow, hyperglycaemia and inflammation. KLF2 has been identified as a mechanosensitive gene, modulated upon exposure to disturbed flow. Desert hedgehog (Dhh) is also a mechanosensitive gene which is involved in essential microvascular integrity signalling and is a downstream target of KLF2 (Caradu et al 2018). Dhh expression in endothelial cells is upregulated by shear stress and downregulated by inflammatory cytokines. Dhh downregulation results in increased endothelial permeability and stimulation meanwhile upregulation prevents TNF-α and glucose induced endothelial dysfunction. In a diabetic, hind-limb-ischaemic mouse model, Dhh-signalling agonist improved endothelial function (Caradu et al 2018).

Approximately 20-30% of all PAD patients have either type 1 or 2 diabetes mellitus (Norgren et al 2007). Hyperglycaemia can bring about intracellular adaptations to the endothelial redox state and induce overexpression of growth factors leading to cell proliferation. There is an increased presence of oxidative stress in diabetes (high ox-LDL levels) promoting a highly inflammatory environment. A review of studies evaluating endothelial function in diabetes revealed a reduced ability of eNOS to produce NO and a close relationship between diabetes and atherosclerosis (Hadi et al 2005). One such way hyperglycaemia may impact endothelial function is through its effects on the endothelial glycocalyx which leads to impaired shear stress vasodilator response. High blood glucose levels can lead to proteoglycan matrix destruction, reduced flow-induced eNOS stimulation and impaired endothelial alignment in response to shear stress.

Glycocalyx shedding can occur in diabetes and atherosclerosis, particularly in response to hyperglycaemia. Shedding occurs upon chronic exposure to inflammatory mediators such as cytokines. Inflammatory conditions promote glycocalyx degradation by activating leukocytes which release enzymes capable of breaking down the glycocalyx structure. Turbulent shear stress can also elicit shedding. During shedding, components of the glycocalyx are lost leading to altered functions including a reduced ability of the glycocalyx to perform its mechanosensing and transduction functions. The luminal barrier of the

endothelium is compromised promoting increased leukocyte cell adhesion and infiltration. Essential enzymes and signalling molecules held within the glycocalyx are lost. Shedding can promote oedema formation, platelet hyperaggregation, loss of vascular responsiveness and accelerate inflammation. All of these consequences make the vascular site significantly more vulnerable to atherosclerosis. ApoE mice with compromised glycocalyx exhibit increased leukocyte adhesion leading to atherosclerosis (Nagy et al 2010).

Regaining endothelial function, and vasodilator capacity, can improve cardiovascular outcome by attenuating disease progression. It is possible to restore endothelial-dependent vasodilation through pharmacological intervention including lipid-lowering therapy, statins and angiotensin-converting enzyme (ACE) inhibitors which reduce oxidative stress and promote vasodilation (bradykinin). Furthermore, diet and exercise as well as glycaemic control have been shown to be effective in restoring vasodilator capacity (Davignon & Ganz 2004). Sedentary behaviour is said to impair endothelial activity and lead to microvascular dysfunction. Physical activity has a positive impact on blood pressure, serum lipids and body mass. Physical activity can also have positive cardiovascular benefits which may be mediated through effects on the endothelium, arterial remodelling and arterial compliance. In PAD, increased physical activity has been shown to increase the frequency and duration of laminar shear stress on the vascular wall, promoting endothelial stimulation (DeSouza et al 2000). Omega-3 has been shown to have multiple effects on the cardiovascular system and studies have proved its ability to improve endothelial function in healthy volunteers as well as high risk cardiovascular patients with atherosclerotic disease. Omega-3 supplementation for three months can reduce circulating pro-inflammatory biomarkers in patients with intermittent claudication (Hammer et al 2019). After three months of supplementation, endothelial function, as measured by flow-mediated dilation (FMD) significantly improved compared to placebo however this effect was not sustained three months post-washout (Hammer et al 2019).

2.2.3 Assessment of the Endothelium

As endothelial dysfunction precedes clinical manifestation of atherosclerotic cardiovascular disease, assessment of endothelial function can be a powerful tool in determining an individual's cardiovascular risk. Early detection of poor endothelial function may be useful

in the diagnosis/prevention of cardiovascular disease. Endothelial function is routinely measured during cardiovascular research as a marker of vascular function however is not currently assessed in clinical practice. Endothelial function and vasodilator capacity can be assessed both invasively and non-invasively by means of intracoronary ACh infusion, flow-mediated dilation, peripheral arterial tonometry and laser Doppler imaging with iontophoresis.

Intracoronary ACh infusion was one of the first methods developed for measuring endothelial function in vivo back in 1986. The technique is highly invasive as it requires injection of a contrast agent into the coronary artery. The vessel diameter can be measured using ultrasound and changes in diameter with ACh infusion recorded. Alternatively, coronary blood flow can be recorded using Doppler as increasing doses of ACh are infused. Endothelial function is calculated by evaluating volumetric coronary blood flow in relation to dose response curve (Premer et al 2019). While healthy individuals show vasodilator response upon ACh infusion, patients with coronary artery disease show dose-dependent vasoconstriction. This method is the most direct measure of endothelial function and is heavily validated. However, it is extremely invasive and expensive as well as requiring a skilled clinical to perform the procedure. This method was replaced by flow-mediated dilation (FMD).

Flow-mediated dilation (FMD) was first introduced as a non-invasive method for assessment of endothelial function in 1992, replacing intracoronary ACh infusion as the gold-standard. During FMD, ultrasound is used to measure artery diameter, usually the brachial artery, at baseline (Figure 2.7). After a short period of ischaemia, the blood vessel diameter is measured again and percentage change in artery diameter is calculated. In healthy individuals, an increase in blood vessel diameter occurs due to reactive hyperaemia. Reactive hyperaemia refers to the increase in blood flow which occurs after a period of ischaemia and is mediated by endothelial NO (Green et al 2014). Therefore, FMD provides a direct measure of endothelial function in the macro vessels. Non-endothelium-dependent dilation (NMD) can be measured, as a control, using a similar protocol however administration of glyceryl trinitrate (GTN) is used as a stimulus instead of reactive hyperaemia. FMD is correlated with endothelial function and is believed to be largely mediated by NO as NO inhibition significantly inhibits the response. Various factors can affect FMD measurement including room temperature, diet, medication, exercise, smoking and stage of menstrual cycle in

women so these variables require tight control. FMD is an independent predictor of CV risk in PAD patients with a low FMD being associated with high risk of lower limb amputation (Brevetti et al 2003). Differences in FMD have not been recorded in PAD with differing stages of the disease suggesting endothelial dysfunction occurs early in disease and is maintained as the disease progresses (Maldonado et al 2009).

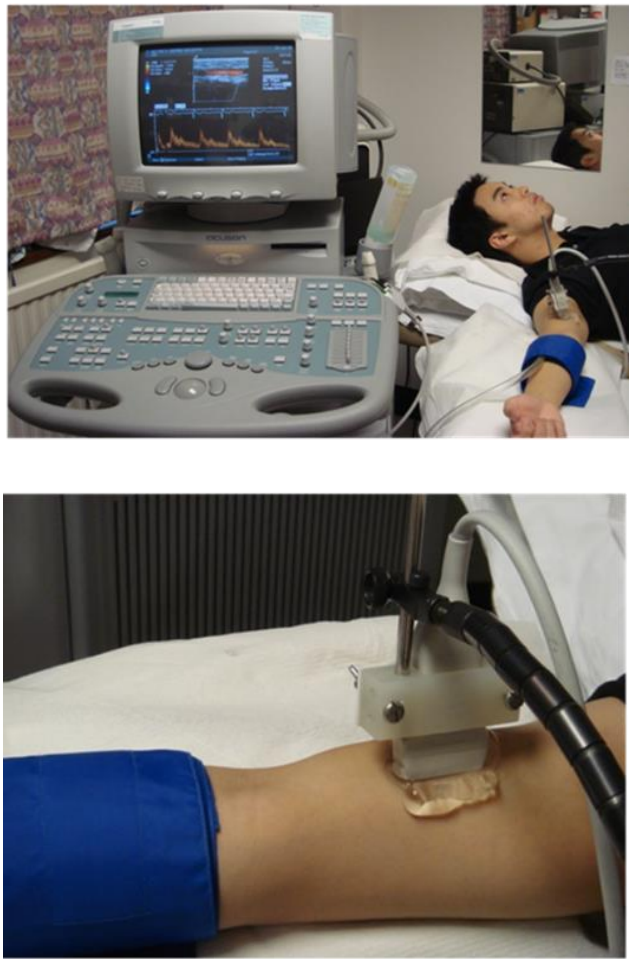

Figure 2.7 ***Flow-mediated dilation (FMD) assessment.*** FMD is considered a gold-standard technique in measuring macrovascular endothelial function. During assessment, the patient is supine with their arm stretched out. The brachial artery is located with an ultrasound device and measurements of the artery diameter taken. A cuff is inflated around the forearm to induce ischaemia for a period of 5 minutes. After cuff release, in healthy physiology, the brachial artery dilates due to NO-mediated dilation. The vessel diameter is measured again and FMD calculated as a percentage change in diameter. FMD = flow mediated dilation, NO = nitric oxide.

While the methods discussed so far look at macrovascular endothelial function, microvascular endothelial function, which is correlated to the central CV system, can also be measured by using peripheral arterial tonometry (PAT) or iontophoresis.

In the early 2000s, Itamar Medical Ltd produced EndoPAT, a novel non-invasive technique to assess endothelial function which offered a quicker, less operator-dependent and cheaper alternative to FMD. EndoPAT makes use of peripheral arterial tonometry (PAT) with the theory is similar to that of FMD. PAT measures skin blood flow in the fingertips following a period of ischaemia using inflatable cuffs and changes in blood flow of the finger arterial bed are measured. The reactive hyperaemia index (RHI) (post-occlusion to pre-occlusion ratio) is calculated by the device. Inhibiting NO during measurement of endothelial function with this method only attenuates the response by 50% suggesting this technique is only partially dependent on NO and vasodilator responses may involve other vasodilators including PGI2 (Bruno et al 2014).

Iontophoresis is commonly used to assess cutaneous microvascular endothelial function. Iontophoresis utilises a small electric current, applied to the skin, to deliver any charged molecule into the skin microcirculation. Normally, blood flow responses to chemical introduction are recorded using laser Doppler imaging techniques. Most commonly, to assess endothelial function, positively charged ACh and negatively charged sodium nitroprusside (SNP) are used. Other vasodilators which can be used include bradykinin and substance P. ACh is a vasodilator agonist and will activate the endothelium to produce and release NO, eliciting vasodilation seen, through laser Doppler imaging, as an increase in blood flow. SNP is an NO donor which is broken down into NO and acts directly on smooth muscle cells to elicit vasodilation and a consequential increase in blood flow. SNP exerts is vasodilator actions independent of the endothelium and is therefore used as a control. Vasoactive chemicals can be diluted in a number of vehicles including deionized water or NaCl.

During iontophoresis, a circular electrode is placed on the skin, usually a hair-free area, and vasoactive drugs can be introduced to the centre of the electrode. The electrode is connected to an iontophoresis controller on which you can change the current and time of delivery which alters the amount of drug delivered. If only one vasoactive drug is to be administered, a reference electrode can be used to complete the circuit however, positively charged ACh and

negatively charged SNP are usually used concurrently. The change in blood perfusion, measured by laser Doppler, is used to calculate percentage change in perfusion from baseline to peak response. In healthy individuals, with functional intact endothelium, delivery of ACh and SNP will both bring about a rise in blood flow. The vasodilator response to ACh is normally greater than that of SNP indicating a healthy endothelium. In diseased individuals, including those with atherosclerotic disease, the vasodilator response to ACh is blunted alongside a normal response to SNP, indicating a dysfunctional endothelium. A blunted response to SNP can be attributed to a reduced vasodilator capacity caused by a structural change in the blood vessel or can be due to reduced NO activity due to high oxidative stress (Turner et al 2008). Jagren demonstrated that iontophoresis administration of ACh and SNP elicits a local vasodilator response in patients with PAD. It was demonstrated that this response in PAD was blunted compared to age-matched healthy volunteers and even more so compared to young healthy volunteers (Jagren et al 2002).

Iontophoresis is non-invasive and allows for quick, easily accessible assessment of the microvascular endothelium. Furthermore, the volume of drug being delivered is small and remains local to the site of delivery with no systemic side effects. The vasodilator responses elicited by this technique are not exclusive to NO and may also be mediated by PGI2. Caution has to be taken in the case of repeated recordings as the skin microcirculation is extremely heterogenous and varied results may be produced at different skin sites. Care has to be taken to place the iontophoresis chambers in the same position as previously used. Furthermore, like PAT, variances can occur due to room temperature, time of day measurement and caffeine intake so these variances need to be mitigated and noted.

2.3 Vasomotion

Blood flow in the microcirculation is not steady, it fluctuates around its steady value in an oscillatory manner and is regulated by interactions between a number of different systems including the sympathetic nervous system, myogenic activity and the endothelium. Together, these systems regulate rhythmic oscillations in the arterial wall, termed vasomotion. Vasomotion is the spontaneous oscillation in tone which occurs in the blood vessel wall and has been linked to oscillations in smooth muscle cell intracellular Ca^{2+} concentrations. Vasomotion is more common in the microvessels, particularly those supplying the skin

(Intaglietta et al 1990 Pradhan et al 2011). Large vessels contract and relax causing changes in blood vessel diameter, as the vessel size decreases the change in diameter becomes faster and greater in amplitude. Vasomotion brings about oscillations in blood flow called flowmotion. This is a characteristic of the arterioles. Evidence has suggested that flowmotion is important in supplying oxygen and nutrients to tissues (Tsai & Intaglietta 1933, Aalkjaer et al 2011). The oscillations in flow represent the influence of six regulatory components in cutaneous circulation which are either central or peripheral; central factors include heartbeat and respiration whereas periphal factors include myogenic, neurogenic/sympathetic, NO-dependent endothelial and NO-independent endothelial activity (Table 2.1).

Table 2.1. ***Vasomotion components.***

	Frequency (Hz)	**Component**	
I	0.6 - 2	Cardiac	The action of the heart ejecting blood into the arterial system creates changes in blood pressure and fluctuations in flow.
II	0.145 – 0.6	Respiratory	Breathing rate may cause fluctuations in flow.
III	0.052 – 0.145	Myogenic	The smooth muscle cells lining the arterial wall contract and relax in response to changes in pressure. The amplitude of this component can be increased by exercise or decreased by local cooling (Shiogai et al 2010)
IV	0.021 – 0.052	Neurogenic	Arteries are innervated by sympathetic nerves from the autonomous nervous system (ANS). The ANS maintains a basal level of arterial contraction. Sympathetic nerves release a number of substances which can alter vascular tone by acting on the smooth muscle including noradrenaline (NA) which promotes vasoconstriction.
V	0.0095 – 0.021	NO-related endothelial activity	Sefanovska demonstrated through iontophoresis of ACh and SNP that oscillations around 0.01Hz originate from endothelial activity (Stefanovska et al 1999). This frequency is said to be NO-dependent as inhibition of NO modulates the component.
VI	0.005 – 0.0095	NO-independent endothelial activity	Perhaps prostaglandin dependent.

Skin blood perfusion mechanisms can be investigated through wavelet analysis of laser Doppler flux (LDF) signals (Figure 2.8). Hafner (Hafner et al 2007) demonstrated wavelet transformation analysis (WTA) can be used to analyse LDF signals which allows for identification of mechanism relating to skin perfusion which may aid in the diagnosis of cutaneous diseases and provide evidence on vascular influences in healthy and diseased states. WTA can be used to identify the six components of flowmotion. WTA allows for the separation of a complex signal into its frequency components. The contribution of each component to the overall signal can then be estimated. WTA has been used to analyse LDF signals obtained from healthy individuals as well as patients with diabetes, hypertension, and myocardial infarction to assess mechanisms of skin perfusion and vascular tone control under healthy physiological conditions as well as in pathological states.

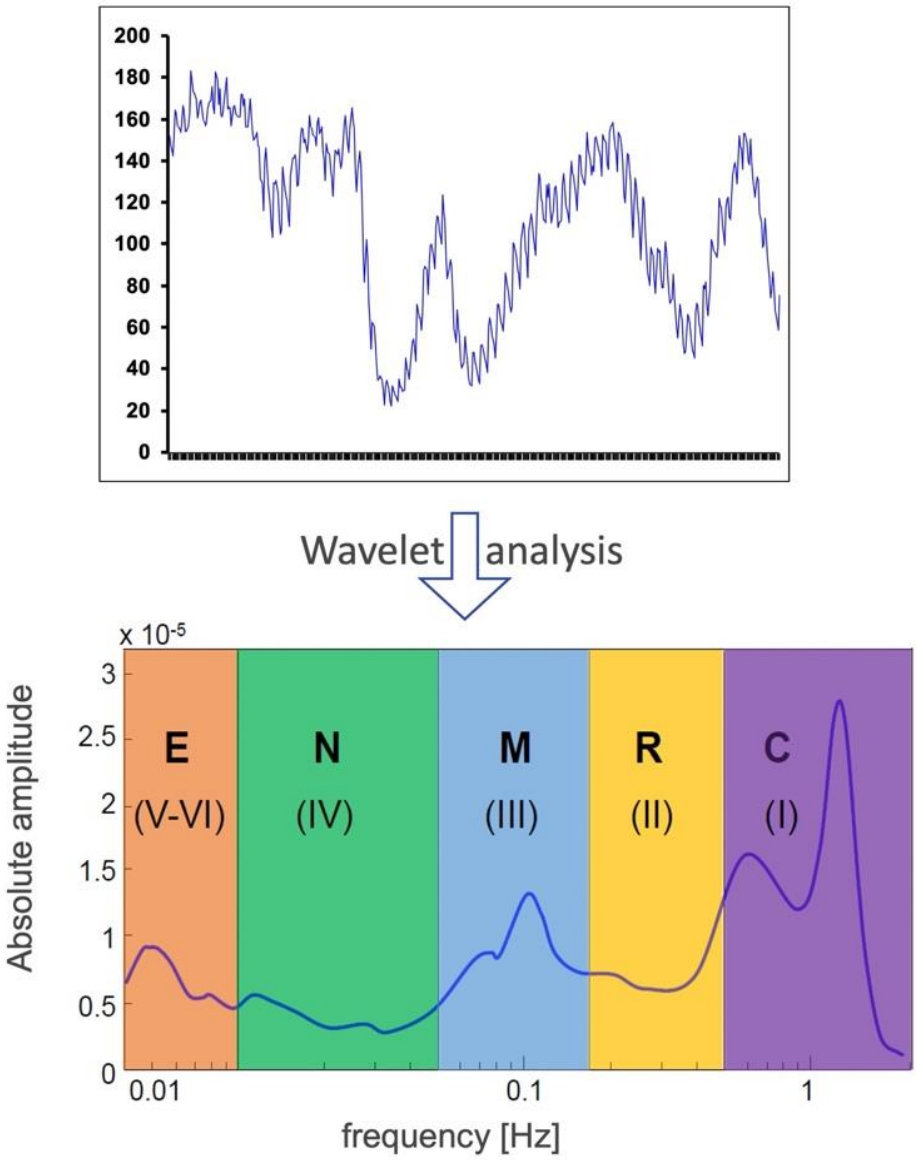

Figure 2.8 ***Wavelet analysis of blood perfusion signals.*** A blood perfusion signal can be obtained through methods including laser Doppler imaging. Within the perfusion trace, small fluctuations in perfusion can be seen which come about as a result of vasomotion. Wavelet transform analysis allows for the separation of the perfusion signal into the six vasomotion components according to their wavelet frequencies. The six components are cardiac (C), respiratory (R), myogenic (M), neurogenic/sympathetic (N), endothelial (E) and non-endothelial (E).

Silva investigated venoarteriolar reflex response to changes in leg position using photoplethysmography (PPG) and performed WTA to assess changes in the contribution of each frequency component. When the foot is lowered, blood accumulates, venous pressure increases triggering the venoarteriolar reflex and a consequential fall in skin perfusion. In this study, the contribution of myogenic, neurogenic/sympathetic, and endothelial activity all

exhibited changes indicating local regulation of the blood vessel was altered. Both cardiac and myogenic activity decreased. The authors suggested cardiac activity may have decreased due to a fall in the relay of blood pressure oscillations to the periphery. A fall in myogenic activity may have been a direct response to an increase in transmural pressure. This study confirmed the use of WTA for flowmotion (Silva et al 2018).

Recordings from laser Doppler show that, as described, microvascular blood flow is not steady, it fluctuates around a steady baseline value (Stefanovska et al 1999). Stefanovska used wavelet analysis to investigate the vasodilator effects of ACh and SNP to determine whether the different vasodilator properties of ACh and SNP is reflected in any vasomotion oscillations. Iontophoresis of ACh and SNP was performed on 9 healthy male volunteers and laser Doppler used to measure blood perfusion responses. As expected, skin blood perfusion significantly increased versus baseline with both ACh and SNP infusion. There were no significant differences for each component between ACh and SNP except for the frequency interval 0.0095Hz-0.02Hz where ACh produced a four times greater response than SNP. The relative contribution of myogenic and neurogenic activity components were decreased by vasodilation. Together, the data from this study provides evidence that endothelial activity is involved in oscillations at 0.01Hz (Stefanovska et al 1999).

The contribution of each component to flowmotion is highly sensitive and changes are observed with a variety of factors. Endothelial, myogenic and respiratory wavelet energies decrease with age while endothelial wavelet amplitudes are greater in young women than young men. It is known that the microcirculation is severely damaged in cardiovascular disease and studies have demonstrated decreased vasomotion present in patients with diabetes and/or arterial disease. Endothelial wavelet amplitude is decreased during iontophoresis of ACh in heart failure patients (Shiogai et al 2010). In patients with severe disease, who exhibit tissue ischaemia, no vasomotion may be present (Intaglietta et al 1990). Evaluation of the contribution of the six components may help researchers determine which components are affected by arterial damage in a variety of disease settings.

One study of healthy volunteers investigated vasomotion responses to reactive hyperaemia. 94 healthy volunteers were recruited and LDF used to assess forearm skin blood perfusion during and after post-occlusive reactive hyperaemia. Post-occlusion there was an average 4-

fold increase in skin blood perfusion. The amplitude of all components (cardiac, respiratory, myogenic, sympathetic and endothelial) significantly increased post-occlusion versus baseline (Tikhonova et al 2010). However, in diseased patients, responses to reactive hyperaemia differ. Bagno and Martini investigated microcirculatory adaptations to reactive hyperaemia in PAD. They obtained laser Doppler fluxmetry recordings from 5 IC and 19 CLI patients at baseline and post-occlusive reactive hyperaemia in the foot. WTA was performed on the laser Doppler signals obtained. With no significant improvements in local endothelial, neurogenic, or myogenic components observed, this suggests early damage to the microcirculation independent of macrocirculation impairment in PAD patients (Bagno & Martini 2015). Another study analysed LDF signals collected from CLI patients to assess the effect of revascularisation intervention on skin blood perfusion. One-month post-procedure the only wavelet component to show a significant change was cardiac activity which increased. This change, alongside lack of alterations in the other components, could be due to chronic cardiovascular risk factor exposure resulting in unreversible microvascular damage and arterial remodelling which cannot be improved with such procedures (Rigo et al 2009).

2.4 Oxidative Stress and Inflammation

While it is common knowledge that endothelial dysfunction is the first step in the development of atherosclerotic vascular disease, oxidative stress and inflammation also play important roles in the early developmental stages of atherosclerosis. This section will discuss what oxidative stress and inflammation are and their roles in the development of atherosclerotic vascular disease.

2.4.1 Oxidative Stress

Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the ability of the antioxidant system to defend against and detoxify these oxidants. ROS are highly reactive molecules which contain oxygen, examples include superoxide (O^{-}_{2}) and hydrogen peroxide (H_2O_2). ROS are highly reactive as they contain an unpaired electron, making them unstable. They seek to stabilise through interaction with other molecules including proteins, lipids and nucleic acid. ROS are present at physiological

levels in healthy cells, produced through normal aerobic metabolism and contribute to healthy cell physiology. At low levels, ROS function as signalling molecules and are necessary for processes such as angiogenesis. ROS levels are controlled by endogenous antioxidants including superoxide dismutase (SOD).

ROS production and/or the antioxidant defence can become dysregulated and in pathological states, production of ROS is increased. ROS, including free radicals and peroxides, can cause cellular damage through oxidation of lipids and proteins within the cell and can induce DNA damage resulting in long-term modifications to the cell environment and behaviour. ROS can alter endothelial cell function through oxidative post-translational modification (ox-PTM) which regulates target protein structure and function. Low levels of oxidative stress can be overcome by the cell, however, high levels of oxidative stress can result in cell death by apoptosis or necrosis.

Oxidative stress contributes to the development of a wide range of diseases in humans including cancer, Alzheimer's, depression and atherosclerosis. Oxidative stress is thought to be involved in the initial development and progression of atherosclerosis and is considered a risk factor for cardiovascular disease. ROS contribute to the development of atherogenesis in many ways. Traditional cardiovascular risk factors including smoking, diabetes mellitus, hypertension and dyslipidaemia promote ROS production and a state of oxidative stress within vascular endothelial cells (Pignatelli et al 2018). ROS oxidise low-density lipoprotein (LDL) cholesterol as well as high-density lipoprotein (HDL) cholesterol thus contributing to foam cell formation and counteracting the protective effects of HDL. Indirectly, ROS can elicit platelet activation promoting thrombosis.

ROS are produced by various oxidase enzymes. In the vasculature, the main producers of ROS are uncoupled eNOS, NADPH-oxidase (NOX), xanthine oxidase (XO), mitochondria and myeloperoxidase (MPO). Leukocytes can also produce ROS. Polymorphonuclear granulocytes (PMN) produce superoxide and can promote atherogenesis through ROS production and promoting cell adhesion.

Under physiological conditions, NOX enzymes are present in the vasculature and are the main source of vascular ROS. NOX isoforms 1, 2, 4 and 5 are expressed in blood vessels.

They produce superoxide and hydrogen peroxide at low levels which contribute to redox signalling. Overactive NOX is implicated in the initial steps of atherosclerosis through high level ROS production (Drummond et al 2011). Superoxide and hydrogen peroxide activate inflammatory pathways, reduce antioxidant capacity and damage proteins, lipids and DNA. In disease, NOX isoforms are upregulated. Endothelial cells cultured with ox-LDL have increased expression of NOX2 (Honjo et al 2008). This is true for PAD patients with higher levels of NOX2 markers and increased levels of NOX2 activity having been demonstrated in these patients, compared with controls (Loffredo et al 2013) (Ismaeel et al 2018). Furthermore, NOX2 is upregulated in tissue after a period of ischaemia (Karim et al 2015). Increased NOX2 activity is associated with reduced NO bioavailability and endothelial dysfunction. NOX2 activity is independently associated with flow-mediated dilation (FMD) in PAD patients (Ismaeel et al 2018). Inhibitors of NOX can reduce atherosclerotic plaque development in high-fat diet mice (Quesada et al 2015). Furthermore, inhibition of NOX2 prevents ROS production and NOX2 KO mice exhibit reduced ROS levels, thus NOX2 inhibitors provide an interesting therapeutic option (Ismaeel et al 2018).

In the presence of cardiovascular risk factors, eNOS essential co-factors are depleted and eNOS becomes uncoupled. Uncoupled eNOS switches from producing NO to producing superoxide. Superoxide interacts with NO resulting in poorer NO bioavailability and consequently a diminished ability of the blood vessel to vasodilate alongside smooth muscle cell proliferation and migration leading to vascular remodelling. Oxidative stress is thought to be partly responsible for the early arterial dysfunction in atherosclerosis through reduced NO bioavailability. A reduced FMD in PAD patients has been associated with oxidative stress furthermore these patients have elevated circulating oxidative stress markers and reduced circulating nitrite and nitrate (Loffredo et al 2007). Nitrite and nitrate levels were both correlated with FMD and oxidative stress marker levels indicating oxidative stress's involvement in reduced NO availability (Loffredo et al 2007).

Myeloperoxidase (MPO) is a member of the peroxidase family and is released from activated macrophages. MPO produces hydrochlorous acid from hydrogen peroxide which is involved in the oxidation of HDL and LDL cholesterol. Furthermore, MPO inactivates NO thus contributes to a reduced vasodilator capacity. MPO has been identified as a marker of oxidative stress. A cohort study in PAD patients found that MPO levels are a strong predictor of cardiovascular events including MI and stroke (Brevetti et al 2008) (Daugherty et al 1994).

ROS promote the further production of more ROS through oxidation of xanthine dehydrogenase (XDH) enzymes to produce xanthine oxidase (XO) (Ismaeel at al 2018). Xanthine oxidase (XO) is another enzyme capable of producing ROS, namely superoxide and hydrogen peroxide as by-products.

Although aerobic respiration produces ROS at physiological levels, dysfunctional mitochondria produce ROS at higher levels. Mitochondria, and in particular the electron transport chain (ETC), can become dysfunctional upon exposure to classic cardiovascular risk factors and high levels of ROS. Metabolically dysfunctional mitochondria lead to a decline in ATP production, defective ETC and increased endothelial cell apoptosis leading to accelerated myopathy (Koutakis et al 2018) (Gardner et al 2019). As a result of decreased blood prefusion at rest, ischaemia-reperfusion injury occurs in PAD upon exertion. Induced ischaemia-reperfusion cycles can lead to increased mitochondrial ROS production causing mitochondrial DNA damage and ETC dysfunction. Changes in the muscle tissue and functional impairment occur alongside accelerated atherosclerosis. PAD patient myofibers demonstrate higher oxidative stress (Koutakis et al 2018). It has been demonstrated that PAD muscle tissue has decreased ETC complex activity, particularly complex I, III and IV versus healthy muscle tissue (Pipinos et al 2006).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor with an essential role in cellular antioxidant system and is commonly referred to as the ‘master regulator’ of the antioxidant response. Nrf2 activity is activated by oxidative stress and also electrophilic compounds. Under physiological conditions, the activity of Nrf2 is muted by the attachment of kelch-like ECH-associated protein 1 (Keap1). Keap1 is a redox sensor and monitors the oxidative levels of the cell. Under physiological conditions, Keap1 targets Nrf2 for ubiquitination and proteasomal degradation. Upon sensing oxidative stress/heightened ROS levels, the ubiquitin ligase activity of Keap1 is inhibited leading to Nrf2 stabilisation, activation and translocation to the nucleus where it promotes target gene expression. Nrf2 promotes the transcription of many genes involved in drug metabolism and antioxidant defence including superoxide dismutase (SOD), glutathione peroxidase (GPX) and NADPH dehydrogenase. Nrf2 also has antithrombotic effects through target gene expression of heme oxygenase 1 (HO-1), glutathione hormone (GSH), glutamate-cysteine ligase catalytic subunit

(GCLC) and glutamate-cysteine ligase modulatory gene (GCLM). While Nrf2 activity is elicited by oxidative stress, it can also be activated by mechanical forces including laminar shear stress which may promote ubiquitination of Keap1. Endothelial cell Nrf2 expression is induced by mechanical forces and can be altered by flow pattern. In this way, haemodynamics can regulate redox balance in endothelial cells. However, shear stress can also increase expression of NOX subunits leading to increased ROS production.

Although antioxidant and antiatherogenic in nature, Nrf2 has been shown to contribute to atherosclerotic development and disease progression (Mimura et al 2015). In the muscle of CLI patients, Nrf2 activation is significantly reduced leading to a consequential reduction in antioxidant protein levels and increased oxidative stress marker levels, compared to healthy tissue (Koutakis et al 2018). Nrf2 KO mice demonstrate reduced levels of cell protection genes alongside heightened levels of drug-toxicity and oxidative stress leading to disease including heart failure and cancer. Nrf2 poses an interesting drug target in diseases with underlying oxidative stress with Nrf2 activators currently being investigated and trialled. Nrf2 activators, bardoxolone methyl and dimethyl fumarate have shown promising results in phase II trials by activating Nrf2 with proven applications in disease including multiple sclerosis (Suzuki et al 2013, Linker et al 2011). Sulforaphane, found in broccoli, has been shown to be an Nrf2 activator leading to gene expression of HO-1 and NADPH dehydrogenase quinone 1 (NQO-1) causing heightened cellular defences (Zhang et al 1992).

Hydrogen sulfide (H_2S) is an endogenous transmitter involved in vasodilation through promotion of eNOS expression and function and increased nitrite reduction to NO. Furthermore, H_2S is an integral part of the inflammatory response and Nrf2 localisation (Koutakis et al 2018). H_2S protects against oxidative stress as it is able to scavenge ROS. H_2S can increase GHS production, decrease NOX activity and overall has antioxidant capabilities. Nrf2 localisation can be increased by H_2S leading to expression of antioxidant genes and activation of HO-1 signalling. H_2S can decrease oxidation of LDL and reduce ox-LDL uptake by macrophages thus helps to attenuate plaque development. Hind-limb ischaemic mice with induced H_2S expression exhibited increased blood flow and capillary density alongside reduced oxidative stress (Wang et al 2010). Serum levels of H_2S in PAD patients are significantly higher than in controls however H_2S bioavailability is reduced in skeletal muscle of CLI patients (Islam et al 2015). Alongside this, NO levels are significantly

lower. The H_2S:NO ratio may be modified in PAD. Higher H_2S levels may be a compensatory mechanism in endothelial dysfunction since H2S can increase NO production (Koutakis et al 2018). In skeletal muscle of CLI patients, there are significant reductions in H_2S bioavailability, Nrf2 activation and antioxidants and increased biomarkers of oxidative stress (Koutakis et al 2018).

Heme oxygenase (HO-1) is an antioxidant enzyme which catalyses the degradation of heme to biliverdin, ferrous iron and CO. Biliverdin is then further converted to bilirubin. Bilirubin is an important antioxidant which functions to prevent lipid oxidation. HO-1 is regulated by Nrf2. A study of 26 PAD patients found that circulating HO-1 is significantly reduced in patients compared to healthy age-matched volunteers (Signorelli et al 2016). Furthermore, HO-1 was found to be an independent predictor of PAD (Signorelli et al 2016) (Koutakis et al 2018). However, HO-1 levels did not correlate with stage of PAD (Signorelli et al 2016).

The vascular wall expresses superoxide dismutase (SOD), an antioxidant enzyme which is part of the antioxidant defence system. SOD is the first line of defence in mitochondria. SOD converts superoxide into less harmful derivatives. Antioxidant defences, such as SOD, are over-come in disease, including PAD (Pipinos et al 2008). In the mitochondria of PAD muscle cells, SOD levels are significantly reduced when compared to healthy mitochondria (Koutakis et al 2018).

The therapeutic effects of antioxidants have been evaluated in clinical trials with varying results. Antioxidants including vitamin E, vitamin C and glutathione have been shown to protect against oxidative damage in PAD with Vitamin E supplementation capable of improving exercise endurance in these patients. Supplementation of both vitamin E and C can attenuate oxidative stress associated with exercise (Koutakis et al 2018). However, other studies have provided conflicting evidence. A meta-analysis of trials investigating vitamin C and E in over 100000 stroke patients concluded that treatment with antioxidants can increase risk of all-cause mortality, compared to controls (Schurks et al 2010).

2.4.2 Inflammation

Inflammation is a non-specific, protective biological response to vascular tissue injury and harmful stimuli, such as oxidative stress. When oxidative stress ensues, inflammatory mediators are released including inflammatory cytokines. Oxidative stress can trigger inflammation through activation of mitogen-activated protein kinase (MAPK), NFkB, NLRP3 inflammasome and secretion of inflammatory cytokines.

Since 1815, inflammation has been proposed as the principal cause of atherosclerosis. In 1858, Virchow discovered inflammatory cells in vascular plaques and in 1908 Osler suggested inflammation is involved in the development of atherosclerosis (Virchoe 1858, Osler et al 1908). In 1999, atherosclerosis was first defined as an inflammatory disease (Ross 1999). Today, it is widely accepted that atherosclerosis is an inflammatory condition. Low grade inflammation contributes to atherogenesis by promoting endothelial dysfunction, formation of atherosclerotic plaque and plaque rupture. Increased inflammatory mediators promote white blood cell infiltration and macrophage activation. Macrophages undergo transformation into foam cells which release more inflammatory cytokines and amplify the inflammatory response. Furthermore, as described previous, inflammation contributes to endothelial dysfunction in numerous ways including reduced NO bioavailability and glycocalyx shedding, as previously described. Furthermore, inflammation is strongly associated with arterial stiffening, which will be discussed later in section 2.4.

Immune cells secrete signalling molecules called cytokines which regulate the immune response. Cytokines can be divided into interleukins (IL), chemokines and tumour necrosis factor alpha (TNF-α). Cytokines are involved in all stages of atherosclerotic development, endothelial activation, monocyte adhesion and infiltration, oxidation of LDL, foam cell formation, plaque formation and plaque rupture. Cytokines promote expression of adhesion and chemotactic molecules by endothelial cells causing endothelial cell activation. Activated endothelium release pro-inflammatory cytokines and thereby further promote endothelial activation. Chemokines are small cytokines released by cells involved in chemotaxis, the movement of cells, and they recruit monocytes. TNF-α promotes ROS production and oxidation of LDL.

Interleukins (ILs) are signalling cytokines which form an essential part of the immune system and inflammatory response. ILs are produced and released by white blood cells during an inflammatory response and allow leukocytes to communicate with one another and coordinate the immune response. ILs can be pro or anti-inflammatory. IL-1β, IL-6 and TNF-α are pro-inflammatory cytokines. IL1-receptor agonist and IL-10 are anti-inflammatory. IL-6 can be either pro or anti-inflammatory. Pro-inflammatory cytokines induce expression of adhesion molecules on the endothelial cell surface which leukocytes adhere to. Furthermore, inflammatory cytokines are released by activated monocytes in the sub-endothelial layer.

Smoking and diabetes are the strongest risk factors for atherosclerosis and arterial disease. Both are known to induce oxidative stress and promote an inflammatory environment. Hypertension, which effects approximately 80% of PAD patients, is also influenced by inflammation (Signorelli et al 2014). Angiotensin-II is a molecule involved in the Renin-Angiotensin System (RAS) and is known to exert effects on the kidney, brain and arterioles with its effects ultimately increasing blood pressure. Angiotensin-II promotes ROS production, pro-inflammatory cytokine expression and VCAM expression on the endothelial surface. Dyslipidaemia activates inflammation and modifies the oxidation of LDL. Together, high blood pressure and cholesterol promote inflammation which itself promotes increased blood pressure and dyslipidaemia resulting in oxidative stress, inflammatory pathways and an atherogenesis environment.

Hypoxia in the ischaemic limb of PAD sufferers initiates inflammatory pathways and tissue damage. Inflammation contributes to lower extremity functional impairment by accelerating atherosclerotic development and disease progression and by adversely affecting the skeletal muscle (McDermott et al 2009). Pro-inflammatory cytokines promote skeletal muscle proteolysis and thereby interfere with muscle repair after injury. They also promote plaque instability and rupture (McDermott et al 2009). Studies on PAD patients revealed increased muscle tissue IL expression after periods of limb ischaemia (Wang et al 2016). Inflammatory biomarkers including IL-6, VCAM1 and CRP are associated with functional impairment in PAD patients as measured by walking velocity and 6-minute walk test (McDermott et al 2008). In a study of 432 PAD patients to determine the relationship between functional impairment and inflammation, it was revealed that higher levels of various inflammatory

markers (including IL-6, VCAM, ICAM and CRP) were associated with a poorer 6-minute walk test. The study concluded that higher levels of inflammation translated to poorer lower extremity functionality (McDermott et al 2008).

C-reactive protein (CRP) is an inflammatory marker produced by leukocytes in the liver in response to inflammatory cytokines, in particular IL-6. CRP levels are closely correlated with systemic levels of inflammation. CRP induces pro-inflammatory cytokine and monocyte chemoattractant protein 1 (MCP-1) release which attracts circulating leukocytes to the endothelium. CRP also induces upregulation of pro-inflammatory cytokines, endothelial adhesion molecules and reduced NO production. Although iNOS is induced by cytokines during an inflammatory response to promote NO production, both iNOS and eNOS require cofactors to function currently. CRP enhances superoxide production which contributes to impaired cofactor BH4 function, NOS uncoupling and reduced NO availability (Jialal et al 2009, Verma et al 2002, Ismaeel et al 2018). CRP is inversely correlated with flow-mediated dilation (FMD) in PAD patients. There is a direct association between low ABPI and high levels of CRP indicating CPR levels increase with disease severity (Silvestro et al 2003) (Folsom et al 2001) (Ismaeel et al 2018) (Stone & Yacoub 2014). PAD patients with highest levels of CRP are most likely to undergo revascularization (Stone & Yacoub 2014). CRP can be used to predict clinical outcome in PAD patients undergoing revascularisation (Signorelli et al 2014) and CRP has been shown to be inversely related to success of angioplasty (Folsom et al 2001, Lin et al 2010). Furthermore, a higher CRP level is associated with increased cardiovascular morbidity and mortality (Stone & Yacoub 2014). Statins can be used to reduce CRP levels and attenuate inflammation (McDermott et al 2009).

The risk of developing PAD is associated with levels of inflammatory biomarkers including CRP and ICAM-1 (Ridker et al 1998, Tzoulaki et al 2007, Ismaeel et al 2018) with PAD patients exhibiting higher circulating levels of inflammatory biomarkers including CRP, IL-8 and VCAM1 (Gardner et al 2014, Vainas et al 2005, Murabito et al 2009, Wildman et al 2005, McDermott et al 2003). The Edinburgh Artery Study found elevated ICAM1 was independently associated with development of PAD but VCAM was not (Tzoulaki et al 2007). Inflammatory markers have been associated with disease severity in PAD as levels of circulating biomarkers are elevated in CLI patients versus those exhibiting intermittent claudication (Cassar et al 2005, Owen et al 2007). A study of endothelial cells cultured from

156 PAD patients found endothelial cell apoptosis is significantly greater than in controls. These patients exhibited lower systemic antioxidant capacity and higher levels of inflammatory markers and vascular function biomarkers including CRP, IL-8, VCAM and VEGF (Gardner et al 2014).

Gardner investigated the effects of exercise on inflammation in PAD patients. 114 patients were assigned to either a supervised exercise programme, home-based programme, or no exercise. Both groups who undertook exercise, supervised and at home, showed decreased endothelial cell apoptosis. At home exercise resulted in increased antioxidant capacity and level of angiogenesis and decreased endothelial-derived inflammation. This study provided strong evidence to show regular, moderate exercise as delivered through exercise programmes can reduce inflammation and attenuate oxidative stress in PAD patients (Gardner et al 2019).

Therapeutic attenuation of inflammation is currently being evaluated. Statins aid in lowering of LDL cholesterol but also have been shown to reduce inflammatory signalling through reductions in CRP levels. Canakinumab is another anti-inflammatory pharmacological agent used to inhibit IL-1beta receptor binding. Canakinumab can reduce the rate of cardiovascular events versus placebo, as tested in a large-scale placebo-controlled trial (Ridker et al 2017). As mentioned, Omega-3 supplementation can reduce circulating inflammatory cytokines (Hammer et al 2019).

2.5 Arterial Stiffness

Endothelial dysfunction is the not the only consequence of oxidative stress and inflammation. Inflammation further promotes arterial stiffening, hardening of the arteries. Arterial stiffening contributes to endothelial dysfunction which itself promotes arterial stiffness, thus a vicious cycle occurs. In this section, arterial stiffness and the pathophysiology of arterial stiffening in relation to inflammation and endothelial dysfunction in disease will be discussed.

2.5.1 Definition of Arterial Stiffness

As previously described, the blood vessel wall is composed of multiple layers. The outer layer is made up of a thick layer of collagen fibres which provide reinforcement to the vessel, helping to maintain vessel strength and integrity. The smooth muscle layer contains elastin. Elastin is a matrix protein which allows for elasticity in response to flow, allowing the blood vessel to regain its shape after stretching.

Arterial stiffness refers to how elastic and distensible the arteries are. Stiffness increases as part of the normal aging process due to changes in the collagen-elastin ratio however arterial stiffening can be accelerated by disease, in particular atherosclerosis. Arterial stiffening occurs due to loss of the Windkessel function which can come about due to collagen deposition, elastolysis and vessel wall calcification. The Windkessel function is essential and buffers the pulsatile ejection of blood which occurs during systole and converts it into steady flow. It helps to dampen fluctuations in blood pressure/pulse pressure. Arterial stiffening changes the physical properties of a blood vessel wall which has functional implications. Stiffer arteries alter the vessel's ability to response to changes in blood flow and pressure.

Compliance (C) describes the relationship between changes in blood volume (V) with varying pressure (P). Veins are 20 times more compliant than arteries meaning these vessels allow for larger increases in blood volume with little increase in blood pressure. Compliance is essential to allow the vessel to expand and contract in response to changes in transmural pressure, the pressure difference across the vessel wall. Arterial stiffening reduces vessel compliance, reducing the vessel's ability to respond to changes in pressure.

Every heart cycle there is a pulse pressure wave generated upon cardiac contraction. This forward/anterograde wave travels into the peripheral arterial system. The speed at which the pulse pressure wave travels is termed pulse wave velocity (PWV) and is dependent on arterial stiffness. Upon bifurcations, areas of reduced elasticity and high resistance in the periphery, due to varying wall properties and changing vessel diameter, the pulse wave is reflected. There are many reflected/retrograde waves in the periphery which accumulate to produce one, large, reflected wave returning to the heart. The reflected wave normally returns to the aorta during diastole and adds to the forward/anterograde wave thus impacting central blood pressure. Adding the reflected wave to the anterograde wave produces a pulse pressure waveform. The timing of the reflected wave arriving back at the heart depends on heart rate,

arterial stiffness, and peripheral resistance. Peripheral resistance is heighted in the presence of atherosclerotic lesions.

Central BP = anterograde PW + all retrograde PWs.

In elderly individuals, or those with atherosclerotic disease, the reflected wave returns to the aorta sooner, during systole, which augments late systolic pressure. Due to augmented pressure, the left ventricle must pump harder causing increased oxygen demand leading to left ventricular hypertrophy and possible heart failure.

2.5.2 Augmentation index (AIx)

Blood pressure differs in blood vessels throughout the body and particular differences lie between the central vessels (aorta) and peripheral vessels, such as the brachial artery. Systolic blood pressure at the brachial arterial is around 14mmHg higher than at the aorta due to pulse pressure (PP) amplification. PP is the difference between systolic and diastolic pressure. While systolic pressure increases in the periphery, diastolic pressure does not exhibit the same increase and may even slightly decrease. Systolic and diastolic blood pressure increase with age however beyond 60 years old, diastolic pressure plateaus and declines slightly with increasing age. PP tends to increase with age since PP = sBP – dBP. PP amplification occurs due to the pulse wave, generated upon cardiac contraction, being reflected upon contact with the periphery (Figure 2.8).

The augmentation pressure is the difference in systolic pressure between the two peaks of the pulse pressure wave. The augmentation index (AIx) is a percentage measure of pressure increase in the periphery and calculated as the difference in systolic peaks of the two waves divided by the pulse pressure multiplied by 100. AIx is composed of the contribution of the reflected pulse wave to central pulse pressure. AIx is dependent on four factors: cardiac cycle, pulse wave velocity, the amplitude of the reflected wave and diastolic blood pressure. AIx is influenced by both blood pressure and heart rate (Wilkinson et al 2000). AIx is inversely related to height and heart rate. Due to the influence of heart rate on AIx, AIx is corrected to a heart rate of 75bpm (AIx@75) which allows for correction of this variable. AIx is not

considered a direct measure of arterial stiffness. AIx is highly correlated/associated with PWV however AIx is not interchangeable with PWV (Yasmin and Brown 1999).

In healthy, young individuals, whose arteries are highly elastic and compliant, PWV is low meaning there is slower propagation of the forward wave, the reflected wave arrives back to the heart during diastole, producing a low AIx value. Meanwhile, in older individuals or those with atherosclerotic disease, vessel elasticity is reduced causing increased PWV and the reflected wave arrives back sooner, during systole, increasing AIx (Figure 2.9). A cross sectional study looked at arterial stiffness, as measured by AIx, in PAD patients and healthy controls. PAD patients exhibited higher AIx than healthy individuals and increased AIx was associated with increased likelihood of PAD (Zahner et al 2017).

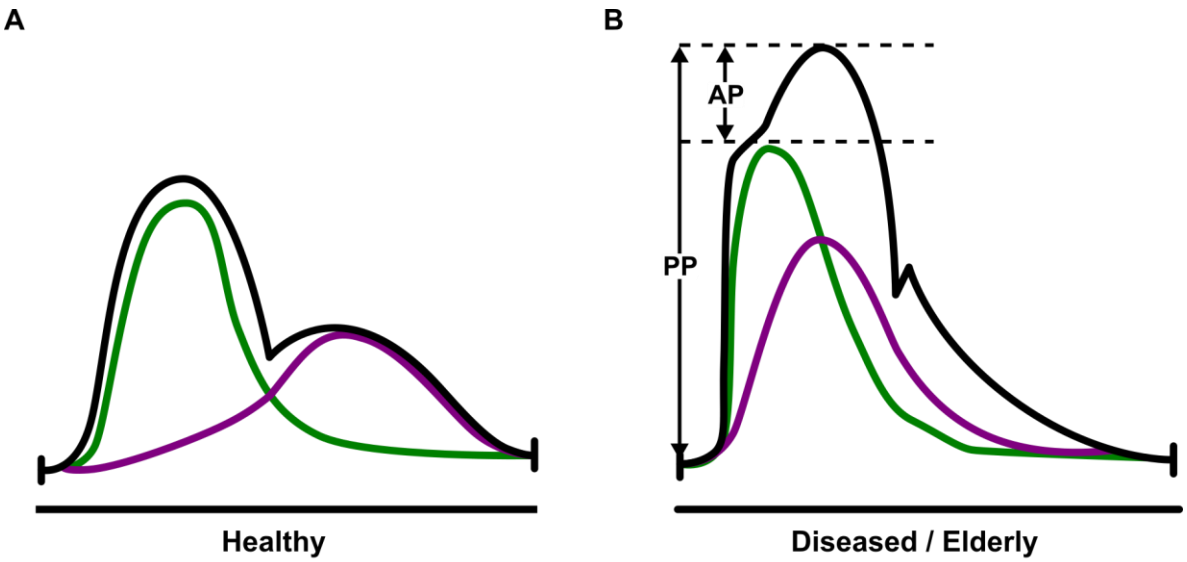

Figure 2.9 ***Healthy and Diseased or Elderly Pulse Pressure Waveforms.*** Green = forward wave, purple = reflected wave, black = combined pulse pressure wave. (A) In healthy individuals, the pressure waveform consists of two distinct peaks, the first one larger than the second which represents the slow propagation of the forward wave which is reflected to the heart during diastole. (B) In diseased or elderly individuals, the reflected wave arrived back to the heart sooner, during systole thus meaning the first peak is smaller than the second in the combined pulse wave. PP = pulse pressure, AP = augmentation pressure.

2.5.3 Pulse wave velocity (PWV)

Pulse wave velocity (PWV) is a direct measure of arterial stiffness. PWV is the speed at which the forward, propagated arterial pulse pressure wave/arterial waveform, which is generated upon cardiac contraction, travels from the aorta through the circulatory system. PWV is calculated by dividing the distance between two points of the arterial system, usually carotid and femoral arteries, by the time taken for the pulse wave to travel between these two points (Figure 2.10). The greater the distance between the two points allows for a more accurate velocity measurement. PWV is highly influenced by vessel distensibility, which is inversely related to stiffness. This relationship is described by the Moens-Korteweg equation (below) where E = the elastic modulus, h = wall thickness, r = vessel radius and p = blood density.

$$PWV = \sqrt{\frac{E\ h}{2\ r\ p}}$$

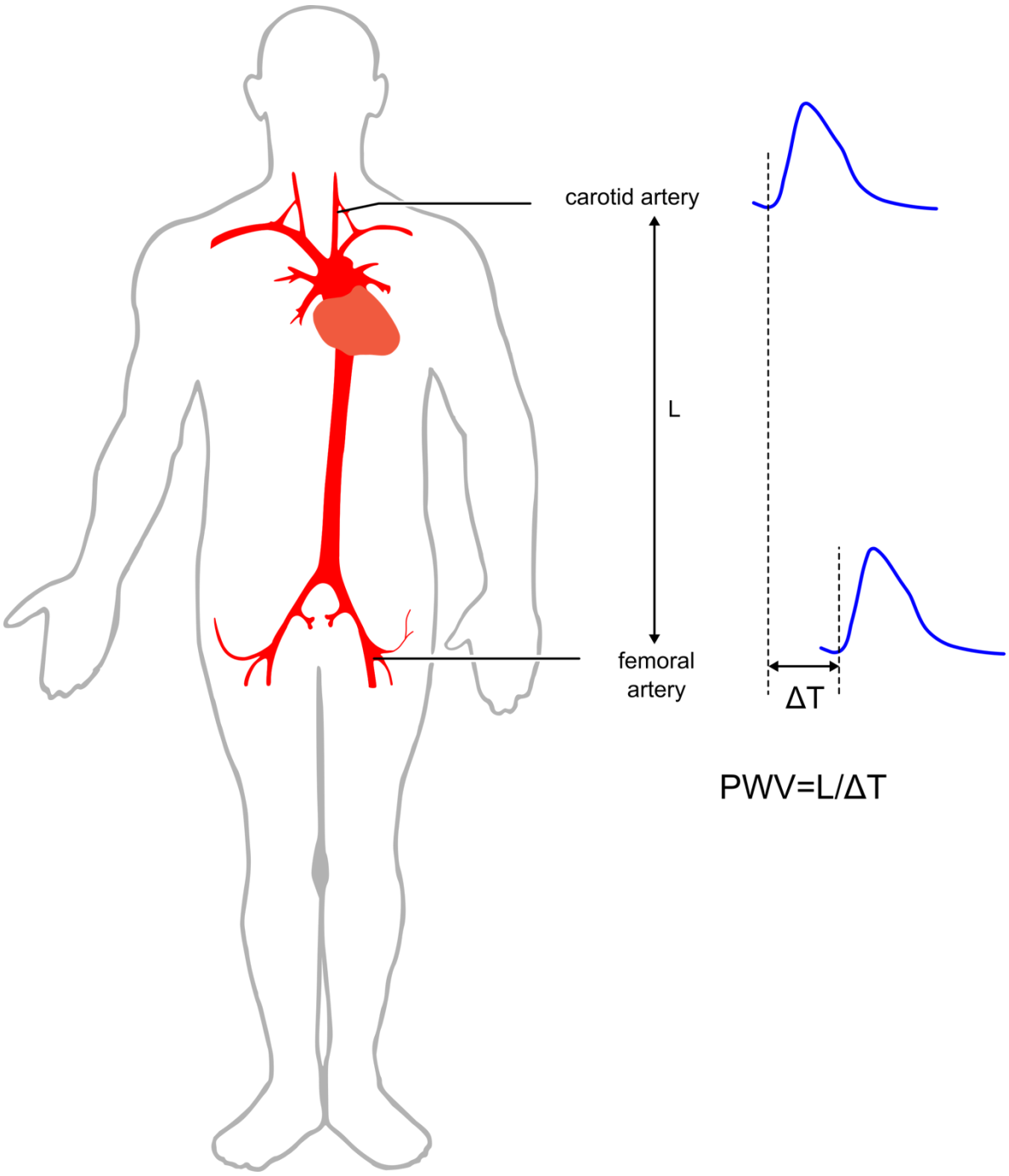

Figure 2.10 ***Pulse wave velocity.*** Pulse wave velocity is measured by capturing the pulse pressure wave at the carotid artery and femoral artery. The distance between these two points is measured (L) and PWV can be calculated as length divided by difference in time between the two points. PWV = pulse wave velocity, L = length, T = time. Adapted from Jeronicic et al 2016.

In highly elastic arteries, such as the aorta, PWV is low as the vessel walls can distend in response to changes in pressure. In arteries with reduced elasticity, typically those with atherosclerotic disease, PWV is high due to reduced distensibility. An average PWV value

for young, healthy individuals is 6m/s meanwhile, in older individuals >70years old PWV is on average 10m/s (Mattace-Raso et al 2010). The Framingham Heart Study found as PWV increases, there is an increased risk of cardiovascular event. An increase in PWV by just 1m/s can increase an individual's likelihood of a cardiovascular event by approximately 14% (Vlachopoulos et al 2010).

2.5.4 Pathophysiology of Arterial Stiffness

The pathophysiological changes that occur leading to arterial stiffness occur during the natural aging process but can be accelerated by chronic exposure to risk factors and disease. These changes include both functional and structural changes to the vessel wall. Atherosclerosis both functionally and structurally promotes arterial stiffness.

Functional changes which occur leading to arterial stiffness occur mostly as a result of inflammation and result in endothelial dysfunction (Figure 2.11). Endothelial dysfunction is the initial step in atherogenesis and promotes arterial remodelling and stiffening. In turn, arterial stiffening promotes endothelial dysfunction which promotes further stiffening, and a vicious cycle ensues. Arterial stiffness is associated with endothelial function. In patients with cardiovascular disease, flow-mediated dilation (FMD) is inversely correlated to central AIx (Soga et al 2008). As arterial stiffness increases, the endothelium can be damaged due to increased haemodynamic load. A cell model was used to determine eNOS activity in both distensible, compliance arteries and stiff arteries. Endothelial cells were grown in-vitro in either distensible tubing, to mimic healthy arteries, or ridged tubing, to mimic older, less compliant arteries. The cells grown in the distensible tubing exhibited phosphorylation of eNOS whereas in the ridged tubing, eNOS activity was not stimulated thus providing evidence that eNOS activity is altered in vessels with reduced compliance (Peng et al 2003). Inflammation also has a significant functional impact on the blood vessel leading to stiffening. Endothelial dysfunction and reduced NO bioavailability is further promoted by inflammation through the actions of pro-inflammatory cytokines, further contributing to a reduced vasodilator capacity. Furthermore, reduced distensibility can affect cyclic stretch promoting oscillatory shear stress. Oscillatory shear stress promotes reactive oxygen species (ROS) production and expression of cell adhesion molecules by endothelial cells thus

promoting endothelial dysfunction and ultimately atherogensis (Janic et al 2014). In this way, arterial stiffness promotes endothelial dysfunction and vice versa.

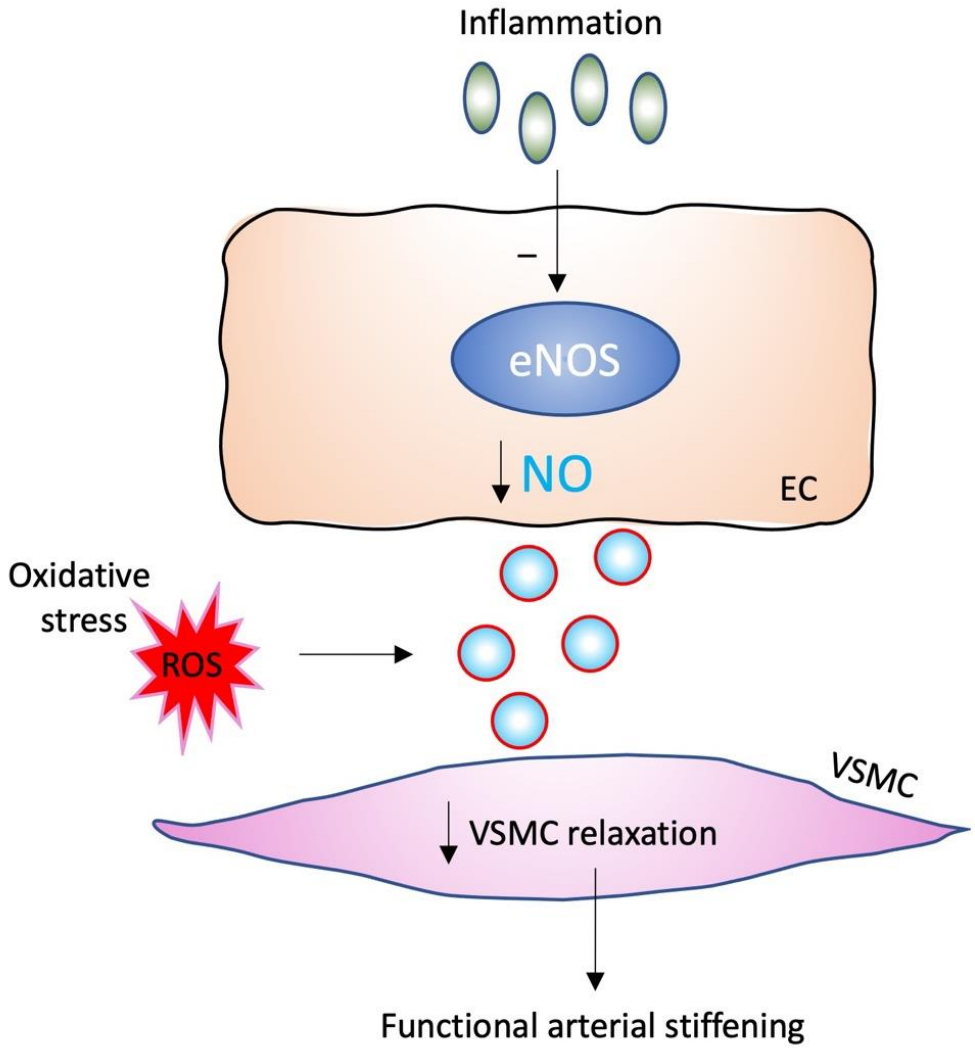

Figure 2.11 ***Functional arterial stiffening.*** Functional arterial stiffening occurs when inflammation promotes endothelial dysfunction, namely inactivation of eNOS therefore causing a reduction in NO production. Oxidative stress promotes ROS redox reaction with NO thus inactivating it. Together, inflammation and oxidative stress promote poor NO bioavailability. As NO is less available and/or active, signalling to promote VSMC relaxation does not occur, and a vasoconstrictive environment is favoured thus stiffening the vessel.

Structural changes in the arterial wall can occur through several mechanisms (Figure 2.12). Aging, as well as atherosclerosis, promotes degeneration of elastic fibres in the arterial wall. Inflammation promotes hyperplasia of vascular smooth muscle cells (VSMCs) and increased collagen synthesis. VSMC phenotype change, from contractile to synthetic, is promoted by

inflammatory mediators, pro-inflammatory cytokines, and promotes leukocyte infiltration and MMP release. MMPs modulate vascular remodelling by increasing VSMC migration to the intima and proliferation as well as increasing the breakdown of elastin and increasing uncoiled, stiff collagen production. Oxidative stress may enhance the effects of MMP as oxidative stress activates MMP and prevents its inhibition. Chronic inflammation promotes VSMC osteoblast marker expression, uptake of phosphate and promotes bioapatite. Together, these inflammatory consequences result in extracellular matrix stiffening, medial calcification, and reduced elasticity. Calcification of arteries make it physically harder for vessels to dilate. These changes lead to the arterial becoming less elastic and compliant and more rigid. In this state, the vessels are less responsive to changes in blood flow and pressure.

There appears to be a strong association between inflammation and arterial stiffness. There are strong correlations between arterial stiffness and inflammatory markers including WBC count, fibrinogen, CRP, and cytokines in many diseases including DM, CAD and PAD (Mozos et al 2017). While inflammatory markers are significantly associated with arterial stiffness in disease, the reverse association has been found in healthy subjects. In experimental settings, activation of inflammation increases arterial stiffness suggesting anti-inflammatory therapy may attenuate arterial stiffness.

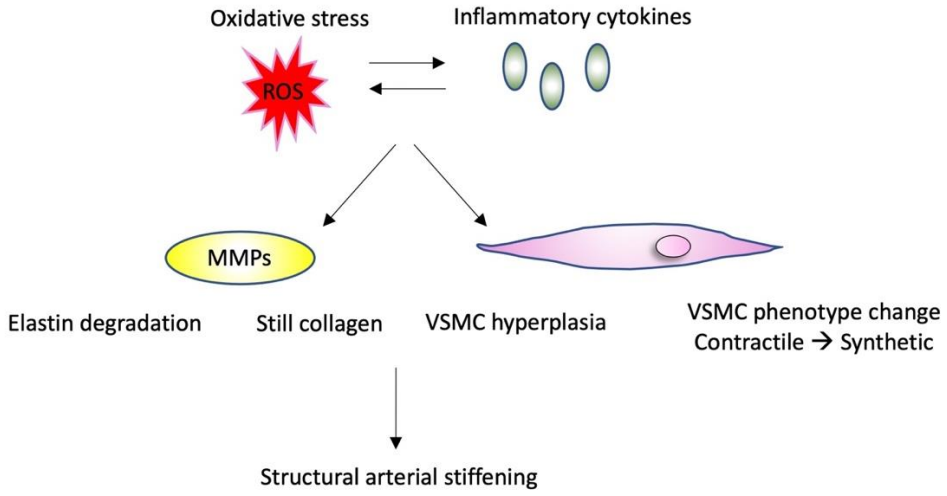

Figure 2.12 ***Structural arterial stiffening.*** Structural arterial stiffening occurs when inflammatory cytokines and oxidative stress promote hyperplasia and phenotypic changes of VSMCs as well as activation of MMP which promotes elastin degradation and stiff collagen production. Together, these inflammatory consequences result in extracellular matrix stiffening, medial calcification, and reduced elasticity. MMP = matrix metalloproteinase, VSMC = vascular smooth muscle cells, ROS = reactive oxygen species.

Arterial stiffness is a strong marker for and is closely associated with cardiovascular risk. The changes in arterial wall structure, which occur during arterial stiffening, and endothelial dysfunction, result in increased SBP and decreased DBP. These changes in systemic blood pressure are likely to have an impact on blood circulation in the heart and brain. In PAD, stiffening is not only limited to the lower limbs but is also systemic resulting in an increased overall risk of cardiovascular events. There have been several studies which have looked at associations between arterial stiffness and cardiovascular disease including links with cardiovascular risk and mortality. Several studies have identified PWV has a predictor of cardiovascular risk including risk of stroke and coronary heart disease. The Framingham study demonstrated a correlation between PWV and development of arterial hypertension within 7 years follow-up (Pinto et al 2019). In PAD patients, reduced arterial elasticity is an independent predictor of cardiovascular and all cause-mortality (Kals et al 2014).

As described, arterial stiffening is directly associated with risk of heart failure. Arterial stiffening can augment late systolic pressure due to the reflected wave arriving later, during systole, meaning less perfusion time for cardiac tissue. Augmented pressure requires cardiac muscles to generate more force for stroke volume resulting in left ventricular hypertrophy which can lead to heart failure. Less perfusion time for cardiac tissue can mean decreased oxygen and nutrient supply and may lead to a myocadiac infarction (MI). Increased arterial stiffness is also associated with brain damage and cognitive conditions such as dementia as elevated arterial stiffness can contribute to cerebral microinfarcts.

Arterial stiffness, measured by cf-PWV is negatively correlated with walking capacity in patients with intermittent claudication. Furthermore, arterial stiffness is positively correlated with age, blood pressure and BMI (Germano-Soares et al 2019). Another study demonstrated similar correlations with lower central AIx associated with longer walking distances in PAD patients (Brewer et al 2007). Revascularisation in PAD has been shown to attenuate arterial stiffness. 3-months post-revascularisation PAD patients exhibited a 10% decrease in central AIx compared to a non-revascularized group in which no change in AIx was observed (Jacomella et al 2012, Silvestro et al 2003).

Arterial stiffness can be improved through pharmacological interventions which include beta blockers, anti-diabetic drugs, angiotensin converting enzyme inhibitors and statins.

Furthermore, lifestyle modifications such as physical exercise and a reduced sodium intake have also been shown to ameliorate arterial stiffness.

2.5.5 Measurement of Arterial Stiffness

Arterial stiffness is associated with and is a good predictor of cardiovascular event risk therefore can be a powerful tool determining an individual's cardiovascular risk. There are both invasive and non-invasive methods of measuring arterial stiffness. Invasive methods, including aorta catheterisation, are not commonly used in clinical practice or clinical research due to their high-risk invasiveness, requirement of medical professionals and high expense and may only be considered when validating a non-invasive method. Non-invasive methods include use of tonometers, oscillometry, ultrasound and magnetic resonance imaging (MRI). The gold-standard non-invasive measure of arterial stiffness is carotid-femoral PWV (cf-PWV), which is considered a direct measure. AIx can be evaluated non-invasively and is closely correlated with PWV however this is not a direct measure of arterial stiffness. Assessment of arterial stiffness is not commonly conducted in clinical practice however is often measured in clinical research to assess macrovascular structure and function.

AtCor Medical (Australia) offer a device, SphygmoCor, which is capable of measuring both PWV and AIx as well as a range of other vascular measures including central blood pressure. The device makes use of applanation tonometry which measures pulse pressure waves, and a pressure cuff which captures the pulse wave through oscillometry. As described previous, PWV is generally measured by assessing the pressure at two sites: the carotid and femoral arteries. cfPWV is the gold standard measurement of larger artery stiffness as it is the most validated, highly reproducible and highly reliable. A tonometer is used to measure the pulse pressure wave at these two sites. The distance between the two sites is measured manually using a measuring tape and PWV calculated automatically by the software. Measuring the distance between the two sites with a tape measure is not ideal as measurements can be inaccurate due to obese patients with protruding stomachs and women with larger busts. Furthermore, this length measuring technique assumes the vessels are connected in a straight line and does not take into account any deviations. The gold standard method for measurement between the two recording sites would be MRI. Older SphygmoCor devices utilised a single tonometer and pulse waves were measured at the carotid and femoral arteries

at separate times. This required synchronised ECG measurement with the R-wave used as a reference to calculate transit time. However newer devices allow for measurement of both carotid and femoral pulse waves in synchronisation with use of both a tonometer (carotid) and pressure cuff (femoral) around the upper thigh. Alam Medical (France) have also produced a device capable of measuring PWV named Complior. This system measures PWV between the carotid and radial arteries by playing clips around the neck and wrist. Unlike the SphygmCor system, which uses tonometers, this system uses mechanotransducers. Although cf-PWV is the gold standard, brachial-ankle PWV (ba-PWV) is sometimes used to measure PWV as this overcomes the need to obtain the femoral pulse which may prove difficult in obese patients with older devices however as newer devices utilise a pressure cuff, this is easier. baPWV correlates with cfPWV however the arterial path between the brachial and ankle arteries is harder to measure to due vessel curvature.

Pressure cuffs, as used by SphygmoCor and Mobil-O-Graph (IEM, Germany), are also used to estimate AIx. These cuffs allow for blood pressure and heart rate to be recorded followed by capture of the pulse pressure wave and estimation of AIx. The system calculates haemodynamic parameters including central aortic pressure, AIx and AIx@75. These devices are non-invasive, have low operator variability, are portable and are extremely quick to perform.

Ultrasound can also be used to assess arterial stiffness in arteries such as the brachial and femoral. Pulse pressure and PWV can both be assessed. To measure PWV using ultrasound, ECG and two transducers are required. However, this technique is extremely challenging and due to operator differences, bulky machines and extensive training required, this method proves less practical. Similar to ultrasound, MRI can be used to measure AIx. Using this method, a variety of arteries can be accessed compared to other methods. To measure PWV with MRI, intravenous contrast MRI is required which is costly, invasive and requires specially trained medical professionals. Photoplethysmography can measure volume changes in the microvasculature and give a pulse wave amplitude estimation. The method is operator-independent, reproducible and can be completed quickly. The measurement correlates with central AIx (Clarenbach et al 2012).

New devices including finger photoplethysmography and 24hr ambulatory monitoring devices which use cuff-based ambulatory oscillometry devices, can be worn during the day to monitor arterial stiffness. These may offer more practical methods for clinical use however these are still in clinical development and require validation.

Chapter 3: Literature Review of Vascular Disease

3.1 Peripheral Arterial Disease

Atherosclerosis present in the arteries peripheral to the coronary and cerebral systems is termed peripheral arterial disease (PAD). Charcot was first to define the disease in the 1850s terming it ‘intermittent claudication’ however it was not until 1982 that critical limb ischaemia (CLI) was clearly defined in publication (Charcot 1958, Bell et al 1982). PAD is characterised by narrowing (stenosis) or complete occlusion of the peripheral arteries. PAD commonly effects the lower limbs but can also be present in arteries of the arms. Aside from atherosclerosis, PAD can also develop as a result of other conditions including thrombosis, fibromuscular dysplasia and arterial spasm.

Sufferers display a plethora of lower extremity symptoms as a consequence of limited blood flow. The most common symptom of PAD is pain and discomfort in the lower body and, as the disease progresses, numbness and ulceration or tissue damage and infection on the legs and/or feet. Ischaemic pain occurs due to lack of oxygen supply and waste removal as a consequence of reduced blood perfusion in the area, creating an ischaemic environment. Broadly, symptoms can be classified into two clinical manifestations: intermittent claudication (IC) and critical limb ischaemia (CLI). IC manifests as muscle fatigue and pain or discomfort in the lower body upon extrusion which can be alleviated upon rest. CLI is a more advanced stage of PAD which is clinically recognised as rest pain and defined phenotypically by ulceration and/or gangrene of the foot. This section will discuss the pathogenesis of PAD, disease risk factors, the symptoms and diagnosis, current treatment options and outcomes.

3.1.1 Pathogenesis of PAD

The pathogenesis of atherosclerotic PAD was earlier thought of as a purely haemodynamic in nature however later research revealed functional impairment involving endothelial dysfunction, inflammation and oxidative stress pathways as the main instigators of atherogenesis leading to PAD.

Atherosclerosis is an inflammatory disease of the blood vessels caused by excessive fat deposition in the blood vessel walls leading to plaque formation and a reduction in artery

diameter (stenosis) or even complete blockage (occlusion) allowing less blood to flow through. The complex process of atherogenesis development is multifactorial, this has been discussed in greater depth in Chapter 2. To summarise, chronic exposure to cardiovascular risk factors results in endothelial damage and activation. Leukocytes, mainly monocytes, adhere to endothelial cells and migrate through into the sub-endothelial space where they are activated by oxidised LDL into macrophages which excrete inflammatory cytokines and proliferate into foam cells with ultimate constitute the plaque (Figure 2.6)..

The process of plaque build-up is slow and often occurs over many years involving a number of events which take place in the arterial wall. Atherosclerosis itself is asymptomatic and may be present in an individual's arteries for many years before symptoms appear due to adaptations in the arteries upon initial plaque formation including the artery increasing in size to accommodate the plaque while still allowing for the same blood flow. Symptoms ensue when a stenosis leads to severe impeding of blood flow or complete occlusion of flow to a tissue.

The most common sites of plaque deposition are at arterial branch points due to the high turbulence in blood flow in these areas. Atherosclerosis of the distal arteries, for example those in the feet and toes, are usually caused by diabetes. Atherosclerosis can give rise to a variety of cardiovascular diseases including coronary artery disease, peripheral arterial disease or ischaemic attacks such as stroke or myocardial infarction. In a 2016 study, it was estimated that PAD was responsible for 26% of global cardiovascular diseases (Bauersachs et al 2019). Atherosclerosis in the coronary arteries may result in symptoms including angina and, if left untreated, can lead to myocardial infarction. Likewise, atherosclerosis in the carotid arteries supplying the brain can produce weakness in one side of the body, difficulty speaking and confusion – symptoms of a stroke which is caused by lack of blood supply to the brain and tissue leading to brain tissue cell death. PAD develops when arteries peripheral to the coronary and cerebral systems are affected including the large arteries which supply the limbs. Arteries of the lower limb, including femoral and iliac, are more susceptible to atherosclerosis than vessels of the upper body (Kroger et al 1999). Around 40-60% of all PAD patients also exhibit coronary and cerebral artery disease (Norgren et al 2007). In individuals with PAD, not including those with CLI, the rate of cardiovascular events

including myocardial infarction and ischaemic stroke is around 5% per year (Campia et al 2019).

In PAD, one or more obstructive atherosclerotic lesions cause a reduced blood flow and a fall in pressure distal to the obstruction resulting in a reduced ankle systolic blood pressure. Upon exertion, flow increases to the working muscle and the pressure difference proximal and distal to the lesion increases thus exacerbating symptoms. Less oxygen rich blood is able to reach the target tissue, possibly leaving the tissue in a hypoxic state leading to multiple dysfunctions causing muscle damage and functional impairment including mitochondrial dysfunction, muscle myofiber damage, peripheral nerve dysfunction and muscle degeneration (Pipinos et al 2003).

3.1.2 PAD Risk Factors & Epidemiology

PAD develops after long-term exposure to cardiovascular risk factors: cigarette smoking, hypertension, age, dyslipidaemia, diabetes mellitus and obesity. Some risk factors are modifiable including smoking and obesity therefore an individual can lower one's risk. However, others are non-modifiable such as age, gender and genetics. Diabetes mellitus and cigarette smoking are two of the biggest cardiovascular risk factors. It has been demonstrated that the more cigarettes smoked, the increased severity of PAD (Norgren et al 2007). One study found that smoking can increase an individual's risk of developing PAD by up to 10-fold, compared to non-smoking (Joosten et al 2012). Diabetes is another big cardiovascular risk factor with diabetics being 88% more likely of developing PAD than non-diabetics and approximately 30% of all PAD sufferers being diabetic (Murabito et al 1997, Fowkes et al 2013). IC is around twice as common in diabetic patients compared to non-diabetic patients (Norgren et al 2007). An individual's risk of developing PAD increases with increasing severity of diabetes mellitus as there a 26% increased risk of PAD with every 1% increase in HbA1c level (Becks et al 1995). Diabetic patients exhibit rapid disease progression compared to those without diabetes. Furthermore, the need for an amputation is up to ten times higher in diabetics than non-diabetics (Norgren et al 2007).

The incidence of PAD increases with age with 5% of 45-50-year-olds being affected, rising to 19% by 85-90 years old (Fowkes et al 2013). Approximately 3-10% of under 70s exhibit

asymptomatic PAD with this figure being 20% in the over 70s (Norgren et al 2007). One third of asymptomatic patients display occlusion of a major artery upon investigation with duplex scanning (Fowkes et al 2013). Differences in prevalence are also present between ethnicities. Individuals of non-white ethnicity appear to have a higher risk of developing PAD, even when adjusted for diabetes, hypertension and obesity. In the USA, by the age of 80, the prevalence of PAD in African American men is double that of men of other ethnicities (Allison et al 2007, Norgren et al 2007, Campia et al 2019).

There is conflicting evidence regarding prevalence in men versus women with number of studies having demonstrated a higher prevalence of PAD in men however more recent studies have suggested the opposite may be true. Women are more likely to be asymptomatic therefore whether the prevalence is higher in one gender than another may depend on which sub-group of PAD patients is being assessed (Patel et al 2020). The 2010 United States census provided information regarding the number of people with PAD and revealed, in the US, more women over 40 years of age had PAD than men. Higher prevalence of PAD in women may be due to oral contraceptives (OC) with those taking OCs having an almost 4 times higher odds of having PAD versus women who do not take OCs (Van Den Bosch et al 2003). Socioeconomic factors also come into play with high income countries have a higher prevalence than low-income countries (Song et al 2019). A low ABPI has been associated with socioeconomic factors including unemployment and low-level of education (Bauersachs et al 2019).

The overall prevalence of PAD has been evaluated in a number of epidemiological studies. The Edinburgh Artery Study is a highly recognised prospective observational study published in 1991 which aimed to determine the prevalence of asymptomatic and symptomatic PAD in the general population. A cross-sectional survey was conducted on 1582 men and women aged 55 to 74 with ABPI measurements obtained. 4.5% of the test population was found to have intermittent claudication and 8% exhibited asymptomatic disease (Fowkes et al 1991). A more recent systemic review of 118 epidemiological studies reporting PAD prevalence between 2011 and 2019 concluded that global prevalence of PAD is 5.56% with approximately 236 million people suffering from PAD worldwide in 2015, up from 202 million in 2010 (Song et al 2019) (Fowkes et al 2013). Overall, it is suggested that the prevalence of PAD is increasing worldwide with an average 20% increase in individual

cases between 2000 and 2010 (Fowkes et al 2013). However, some conflicting evidence suggests PAD prevalence is decreasing (Cea-Soriano et al 2017).

3.1.3 Symptoms, Diagnosis and Classification of PAD

Atherosclerosis develops silently over many years and therefore PAD can manifest with and without symptoms with half of all sufferers being asymptomatic (Hirsch et al 2006). Symptoms can vary depending on the location of stenosis. The most common symptom at first presentation is IC which causes pain and cramping in the limb upon exertion and is relieved with rest. These symptoms ensue due to an increase in oxygen demand by the calf muscles during walking/exercise which cannot be met due to the obstruction in flow. The muscles are then unable to meet the physical demands. Around 80% of IC sufferer's condition remains stable over a 10-year period (Hirsch et al 2006). However, for 20% of IC patients, the disease progresses to CLI. CLI is defined as chronic ischaemic rest pain which ensues for a period longer than two weeks with possible wounds or gangrene due to medically diagnosed arterial disease. CLI is characterised by pain in the leg while at rest due to blood supply being so limited, the muscles cannot be supplied with enough oxygen to maintain resting metabolism. CLI is usually present alongside tissue loss and/or gangrene due to the ischaemic conditions promoting infection and not allowing for wound healing (Figure 3.1). These wounds can be managed and cared for to prevent infection however prove very hard to heal completely as a sufficient blood supply is required to deliver the appropriate nutrients and inflammatory molecules. Wounds are of particular concern in PAD patients as poor management can lead to infection causing further complications and possibility of hospitalisation and/or amputation. Not all patients progress through PAD from asymptomatic to IC and finally CLI. There may be a subgroup of CLI sufferers who are asymptomatic. These individuals are usually sedentary and exhibit peripheral neuropathy reducing their pain sensitivity, this is common in diabetic patients. Patients who are immobile may not experience symptoms until the disease has developed to CLI, and they begin to experience pain while at rest. In these cases, first clinical presentation is CLI, bypassing the opportunity to treat at IC stage.

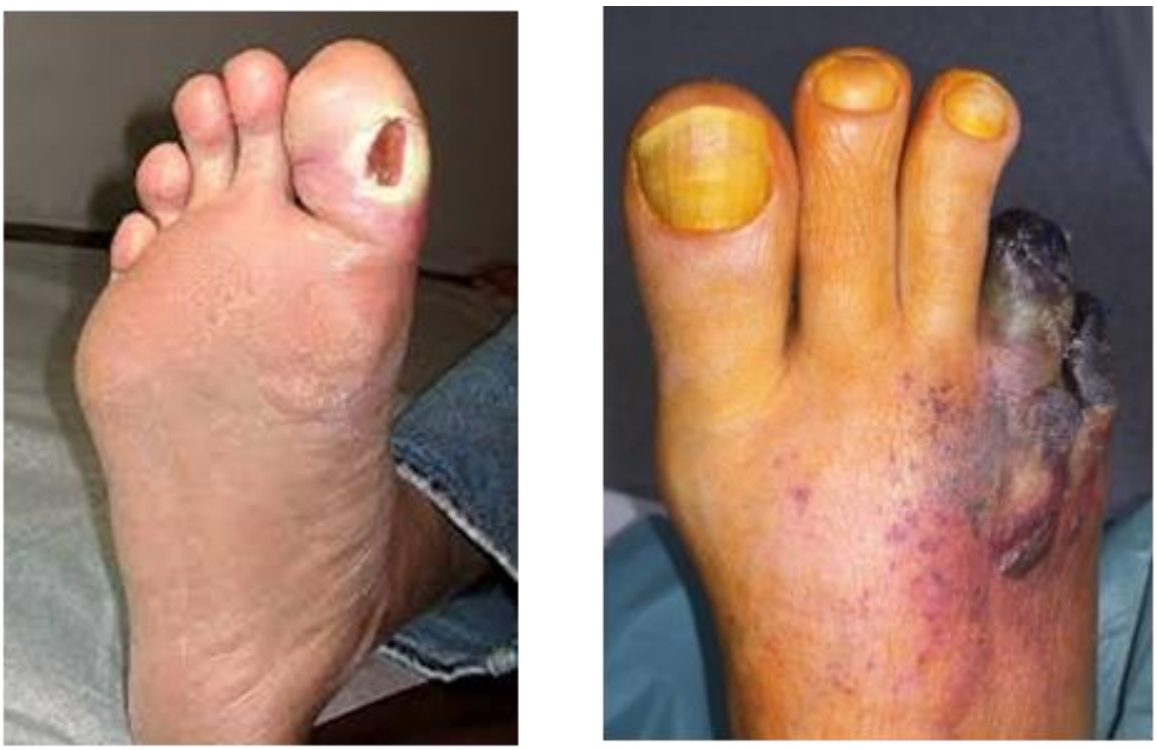

Figure 3.1 ***Critical limb ischaemia ulceration and gangrene.*** Patients suffering from the most severe form of peripheral arterial disease (PAD), critical limb ischaemia (CLI) can exhibit tissue loss in the form of ulceration and/or gangrene.

PAD can cause emotional, social, physical and even financial difficulties for individuals. PAD patients typically have a reduced quality of life due to difficulties carrying out everyday tasks as a result of pain. Having a reduced walking capacity leads to chronic muscle adaptations, fatigue and contributes to an overall reduction in cardiovascular health. Furthermore, the reduction in mobility can affect an individual's ability to work thus resulting in financial impacts and restrict the patient's ability to get out and socialise. Quality of Life (QoL) assessments are routinely used to measure such effects of PAD in patients.

Diagnosis begins with identifying relevant symptoms and reviewing the medical history followed by a physical examination. The leg may be checked for muscle wasting and signs of ulcers or chronic wounds. The foot is examined for a change in skin colour, usually blueing, or texture and a decrease in temperature compared to the opposite foot. Pulses in the foot are routinely checked, including the dorsalis pedis. Whether the individual is a current or previous smoker and suffers from diabetes is also important to note. Care is taken not to confuse leg pain with other conditions including spinal stenosis, osteoarthritis, neuropathy

and venous insufficiency. Buerger's Test is carried out to assess arterial sufficiency. Buerger's disease effects the peripheral arteries and can reduce blood flow, like atherosclerosis, often having the same presenting symptoms. The test is carried out to rule out this disease and involves raising the leg by 90 degrees while supine and noting any changes in colour. Pallor may ensue when Buerger's is present.

If PAD is suspected after physical examination, ankle-brachial pressure index (ABPI) will be measured. ABPI is "the ratio of systolic blood pressure at the dorsalis pedis artery of the foot to the systolic blood pressure of the brachial artery in the arm" and is discussed more in depth in Chapter 4 Methodology. An ABPI <0.9 is indicative of PAD while an abnormally high ABPI also indicates cardiovascular abnormalities. An ABPI of >1.3 indicates stiff, non-compressible arteries caused by calcification thus providing a false negative result in the diagnosis of PAD. This is common in patients with diabetes. Due to this, the diagnosis of PAD can be tricky as stenosis may or may not be present. In this case, Doppler waveform analysis or toe-brachial index (TBI) can be used to detect PAD (Aboyans et al 2012). TBI can be used in situations of calcified larger arteries as the small digital arteries are less likely to be affected by arterial calcification. CLI is medically diagnosed when the ankle pressure <50mmHg or toe systolic pressure <30mmHg. If patients have gangrene and/or wounds, then an ankle pressure <70mmHg or toe pressure <50mmHg is indicative of PAD (Uccioli et al 2018). If classic symptoms are present but resting ABPI is normal, a walking ABPI test may be required in which ABPI is measured both at rest and again after a period of walking until pain ensues. PAD is diagnosed in this way if the ABPI ratio decreases by 15% or more (Norgren et al 2007).

Once initial tests are complete and PAD is suspected, the patient is referred to secondary care for further examination. Doppler ultrasound is used to locate the site of stenosis and extend of disease. It is performed in all patients diagnosed with or suspected to have PAD to evaluate the correct course of treatment. High frequency ultrasound waves are used to measure the blood flow through the arteries and obstructions to flow can be identified. Contrast angiography can also prove a useful tool in identifying arterial obstructions however is a more invasive procedure during which a catheter is inserted into the common femoral artery and a contrast agent injected to visualise blood flow on an x-ray. Before intervention,

magnetic resonance (MR) and computed tomography (CT) angiograms are routinely performed to gain detailed pictures of the blood vessels and identify blockages.

The Fontaine and Rutherford classification systems are the most common ways of classifying PAD (Fontaine et al 1954, Rutherford et al 1997). The Fontaine classification system categorises PAD into 5 different stages ranging from asymptomatic to gangrene (Table 3.1). Meanwhile, the Rutherford classification system categorises the disease into 4 grades (0 – III) and 7 categories (0-6) (Table 3.2). The Fontaine and Rutherford classification systems have been criticised for not being specific enough. Therefore, the Society for Vascular Surgery published the Wound, Ischaemia and Foot Infection (WIFI) classification system which grades CLI patients according to an individual score for wounds, ischaemia and foot infection (Mills et al 2014). With 3 categories being graded, 64 classes are possible, giving a much more accurate classification. The presence of gangrene increases the risk of amputation versus ulceration therefore it is important to take note of (Mills et al 2015).

Table 3.1 ***Fontaine classification of PAD***. The Fontaine classification system characterises PAD in 5 stages; I, IIa, IIb, III and IV.

Stage	**Clinical Presentation**
I	Asymptomatic
IIa	Mild claudication
IIb	Moderate to severe claudication
III	Ischaemic rest pain
IV	Ulceration or gangrene

Table 3.2 ***Rutherford classification of PAD.*** The Rutherford classification of PAD splits different clinical presentations into 4 grades (0, I, II and III) and 7 categories (0-6).

Grade	Category	Clinical Presentation
0	0	Asymptomatic
I	1	Mild claudication
II	2	Moderate claudication
	3	Severe claudication
III	4	Ischaemic rest pain
	5	Minor tissue loss (ulceration)
	6	Major tissue loss (ulceration / gangrene)

3.1.4 Current Treatment Options for PAD

PAD cannot be cured, and treatment depends on a variety of factors including severity of disease, general health and quality of life of the patient. Treatment aims to augment blood flow which in turn provides symptom relief, curbs disease progression and decreases the risk of cardiovascular mortality (Burns et al 2003). Current treatment options range from supervised walking exercise and modification of risk factors to medication and, in patients suffering from CLI, intervention may be recommended such as open and endovascular surgery to avoid major limb amputation (Figure 3.2) (Table 3.3).

Current first line non-interventional treatment options for PAD patients, particularly those with IC, initially involves lifestyle changes such as walking exercise, reduced fat diet and smoking cessation. Smoking cessation is strongly recommended to all patients as it is the strongest modifiable risk factor for PAD and other cardiovascular diseases. Around 1 in 3 PAD patients are active smokers with many more being former smokers (Campia et al 2019). Counselling and medication including nicotine replacement therapy are readily available for

patients however despite this, around 80% of patients trying to quit give up within the first 6 months (Campia et al 2019).

Walking exercise in those who experience mild claudication allows the body to adapt to the blood supply demand by creating new, collateral vessels. Exercise may not be recommended in patients with comorbidities such as chronic obstructive pulmonary disease (COPD). Supervised exercise programmes aim to increase maximal pain-free walking time and/or distance. The ERASE trial compared the effects of supervised exercise therapy alone versus alongside endovascular intervention in PAD patients (Fakhry et al 2015). Walking distance and quality of life were significantly improved in the exercise alone group at one year follow up. However, both parameters improved more in the combined therapy group. The ACC and AHA Joint Task Force recommended supervised exercise programmes as primary treatment for patients with IC (Gerhard-Herman et al 2016). However, uptake of these programmes is poor, and compliance is ever poorer with many elderly patients claiming the programme is inconvenient or too intensive. Approximately 15% of patients taking part in supervised exercise programmes drop out (Hotta et al 2019). While exercise programmes have been shown to improve leg symptoms and cardiovascular risk in patients, exercise may not always be beneficial. Short bouts of strenuous exercise may elicit ischaemia-reperfusion injury, which is known to increase oxidative stress, elicit mitochondrial dysfunction and induce changes in myocyte metabolism and apoptosis causing changes in muscle fibre type, contractibility and atrophy thus contributing to functional impairment (Koutakis et al 2018) (Campia et al 2019).

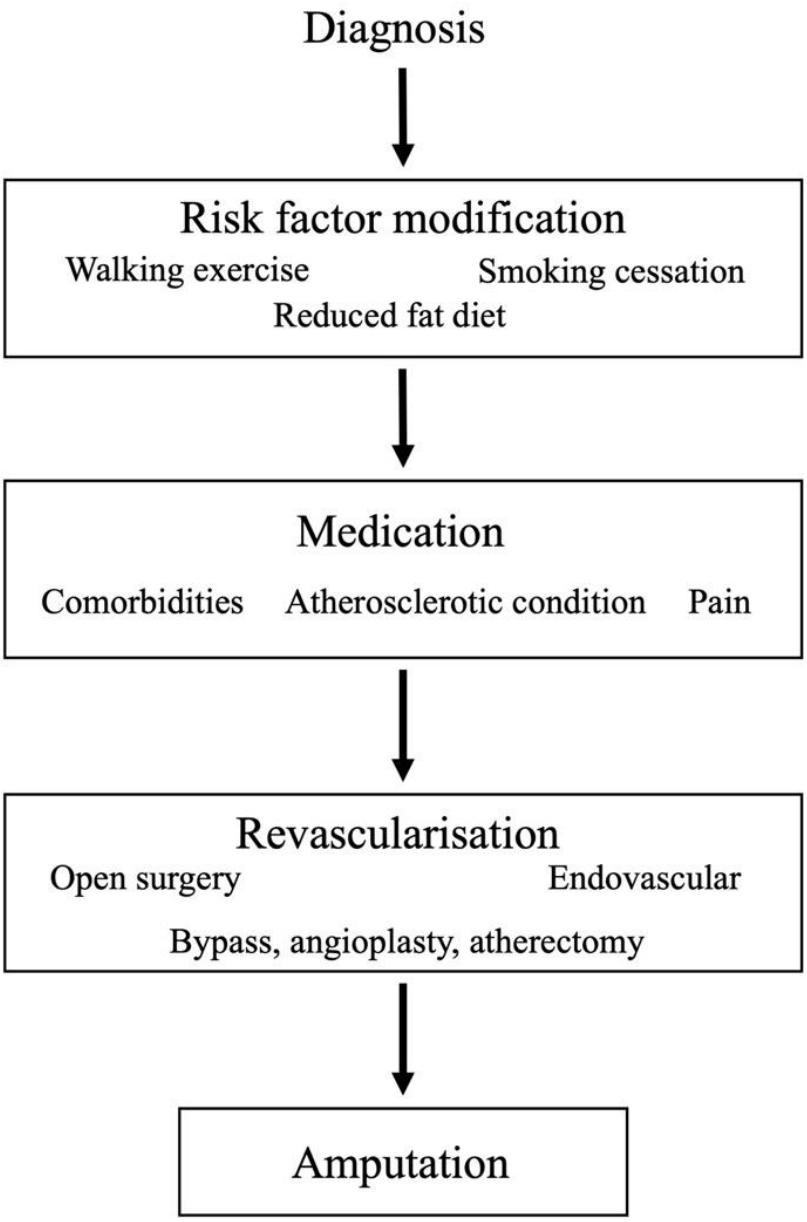

Figure 3.2 ***Current treatment options for PAD.*** Upon diagnosis of PAD, treatment options depend on severity of disease. If disease is mild, risk factor modification may be the first line of treatment which involves walking exercise, smoking cessation and following a reduced fat diet. As the disease worsens, pharmaceutical options can be prescribed including medication to treat any comorbidities, pain, and the atherosclerotic condition. When PAD develops to CLI, revascularisation in the form of open or endovascular surgery is the first line of treatment options. In cases where the limb cannot be salvaged or revascularisation is unsuccessful, amputation may be considered.

Remote ischaemic conditioning (RIC) is a non-invasive technique involving an extremity being exposed to ischaemia used to prevent tissue damage as a result of ischaemia-reperfusion injury based on the theory that intermittent cycles of ischaemia and reperfusion

can be cardioprotective. Studies have shown RIC to improve endothelial function and reduce platelet activation and thus is capable of reducing infarct size in myocardial infarction (Manchurov et al 2014). A small number of studies have investigated the effects of repeated RIC treatment at home. In healthy individuals, RIC can improve endothelial function and microvascular circulation (Jones et al 2014). In a double-blind, placebo-controlled, randomised trial of 36 T2DM patients with PAD, who received RIC to their upper arm once daily for 12 weeks, no significant differences in transcutaneous tissue oxygen tension or PWV were observed post-treatment versus those who used a sham device suggesting RIC may not be beneficial in PAD (Hansen et al 2019).

The use of daily passive stretching has been shown to be effective in enhancing endothelium-dependent vasodilation and blood flow during exercise in rats (Hotta et al 2019). In a randomized, non-blinded, crossover study of 13 symptomatic PAD patients who received 4 weeks of passive calf muscle stretching or no intervention, an improvement in endothelial-dependent vasodilation was recorded, as measured by popliteal FMD, and a significant increase in 6 minute walk test (6MWT) in the intervention group. No change in endothelium-independent vasodilation was recorded through nitroglycerin induced vasodilation post-stretching. 6MWT was positively correlated with FMD and was maintained 4 weeks after stretching cessation. This small-scale study suggests passive muscle stretching may offer a novel therapy for elderly PAD patients and large-scale studies are warranted (Hotta et al 2019).

Medication is used in PAD to manage several aspects of the disease including the atherosclerotic condition, pain and comorbidities. Pain management is essential in all PAD patients to improve quality of life. Pain relievers commonly prescribed include paracetamol, codeine, aspirin or morphine. When pain is neuropathic, a different therapeutic approach is considered. To manage the atherosclerotic condition, statins, ACE inhibitors and antiplatelet drugs are also often prescribed. Antiplatelet therapy, such as aspirin or clopidogrel, prevent thrombosis in arterial disease. Statins, including simvastatin, are lipid-lowering medications which lower LDL cholesterol levels and are proven to attenuate cardiovascular risk and improve endothelial function through increased NO synthesis, ET-1 downregulation and by promoting growth factor expression leading to angiogenesis (Hassanshahi et al 2019). Vasodilators including cilostazol are prescribed with the aim of enhancing vasodilator

capacity. Cilostazol is a phosphodiesterase inhibitor with vasodilator and antiplatelet properties which has been shown to increase walking distance by approximately 50% in some patients (Campia et al 2019). Comorbidities including hypertension, dyslipidaemia and diabetes must be managed in order to reduce risk of secondary cardiovascular disease and/or event. Close control of blood pressure is achieved through prescription of ACEs, ARBs, CCBs, beta-adrenergic blockers and thiazide diuretics. Blood pressure lowering can be achieved by angiotensin-converting enzyme inhibitors such as ramipril and angiotensin receptor blockers. In patients with CLI, taking blood pressure lowering medication significantly lowers their risk of cardio- and cerebro- vascular events (Armstrong et al 2015). In diabetics, glycaemic control is essential to attenuate the progression of vascular complications. Metformin and other diabetic drugs are taken to manage the diabetic condition. Poor glycaemic control in diabetic PAD patients has been shown to limit ulcer healing (Uccioli et al 2018).

Various alternatives to conventional treatment are available on the market. Leg compression devices have been utilised as a means of treating a variety of vascular conditions. These devices can often be purchased low-cost from pharmacies, supermarkets and online. Originally designed to prevent deep vein thrombosis and varicose veins, these devices squeeze the leg to facilitate venous return. Such devices come with several drawbacks and are not routinely recommended in PAD patients. Patients with broken skin, such as those with chronic ulcers, are unsuitable for compression device use as the device itself comes into direct contact with the skin which may promote wound infection. There is a current lack of large-scale clinical trials testing this treatment modality in PAD patients and therefore no data surrounding the clinical benefits of such treatment. Neuromuscular stimulation devices such as transcutaneous electrical nerve stimulation (TENS) machines induce sensory stimulation and are mainly used for analgesic effects. In a study of 40 diabetic claudicants who administered calf muscle electrostimulation of varying frequencies (1-250Hz) on both limbs for 1 hour daily for 12 weeks, a significant increase in absolute claudication distance was recorded (Ellul et al 2020). Similarly, neuromuscular electrical stimulation (NMES) has been shown to elicit vasodilation and increase pain-free walking distance (Loubser et al 1988, Anderson et al 2003). However, like pneumatic compression devices, these methods have not been validated in large clinical trials and are not routinely used clinically in the treatment of PAD.

Negative pressure wound therapy (NPWT) is routinely utilised in clinical practise to aid in chronic wound healing. NPWT is discussed in more detail in Chapter 3. Some chronic wounds can be treated with dermal replacement therapy, such as Dermagraft ®, which aims to deliver growth factors needed to aid in wound healing (Newton et al 2002).

For CLI, the first line of treatment is revascularisation which can be endovascular or open surgical revascularisation however this is dependent on extent of disease, comorbidities and risk of procedural-related complications. Intervention is not an option for all patients with only half of all patients being suitable for surgical intervention and for some, primary treatment may be amputation (Norgren et al 2007). Intervention, both open and endovascular, comes with risk of significant peri operative morbidity and mortality, and in some instances no such intervention is achievable with the patient then faced with amputation or palliation. If left untreated, CLI is ultimately limb and life threatening.

Revascularisation can be achieved through several options which utilise open surgery or endovascular techniques including angioplasty, atherectomy or vascular bypass. For CLI, the first line of treatment, in those who are suitable, is endovascular therapy (EVT). Revascularisation aims to increase blood flow to the ischaemic area with the goal of decreasing rate of limb loss and increasing QoL and long-term survival. This can be achieved through surgical bypass or endovascular therapy (balloon angioplasty). Angioplasty is performed on the large arteries of the limb including the femoral and iliac. The procedure involves a catheter being inserted into the effected vessel, a balloon is inflated to open up the blocked artery and increased blood flow. A stent is then placed into the artery in order to keep it open in the long-term. During bypass, either a blood vessel from elsewhere in the patient's body, usually the saphenous vein, or synthetic blood vessel is grafted onto the affected artery, bypassing the blockage. However, if there are no lifestyle modifications, plaque may also develop in the new artery as it is exposed to atherogenic risk factors. Surgeons can also scrape away plaque that has built up in the artery in a procedure known as atherectomy, with the aim of reducing blockage to blood flow. However, this method yields similar results as angioplasty with little long-term benefit (Ambler et al, 2014). Smoking may increase the chances of a failed revascularization (Uccioli et al 2018).

Survival rates and QoL post-intervention are similar for surgical and endovascular therapies (Hassanshahi et al 2019). Endovascular methods are advantageous over surgical methods due to lower costs, lower mortality and morbidity and a shorter period of hospitalisation being required. As bypass requires open surgery, the perioperative risk of cardiovascular event infection is higher (Hassanshahi et al 2019). The Bypass Versus Angioplasty in Severe Ischaemia of the Leg (BASIL) trail compared bypass versus angioplasty as a primary treatment in CLI (Adam et al 2005). There were no differences in amputation-free survival, all-cause mortality or health related QoL. Morbidity and hospital costs were higher in the surgical group versus those receiving endovascular intervention however those who underwent a bypass survived for longer than the endovascular group (Ucccioli et al 2018).

When all other interventions have been exhausted, ischaemic tissue is unmanageable and the limb cannot be salvaged, amputation is the only option. Minor amputations may be performed to remove part of an ischaemic foot or major above or below knee amputation may be required. If a major limb amputation is to be undertaken, below knee amputations are preferred as it allows preservation of the knee for increased mobility post-rehabilitation. However, the knee cannot always be salvaged. The ratio of below to above knee amputations is 1:1 (Norgren et al 2007). Post-amputation, only 1 in 5 amputees regain full mobility with use of a prosthesis (Dormandy et al 1999, Taylor et al 2005). It is extremely important to prevent an amputation due to the mortality risks associated with the procedure. In a Dutch study of CLI patients, mortality for amputees at 5 years post-procedure was 85% compared to 61% for those who never underwent an amputation (Duff et al 2019). Not all patients who require an amputation steadily progress through each stage of PAD, therefore prevention of such procedures is difficult. Around half of patients who require a lower limb amputation did not exhibit ischaemic symptoms 6 months previous (Norgren et al 2007).

Novel approaches including gene therapy and stem cell therapy have been developed in recent years to overcome the limitations of current treatments. They mainly work by promoting angiogenesis through delivery of proangiogenic factors to trigger formation of collateral flow to the ischaemic area. Stem cell and gene therapies work by either eliciting endothelial activation or migration of stem cells to the affected area. Gene therapy involves a viral or nonviral vector as mode of delivery (Hussanshahi et al 2019).

Hyperbaric oxygen therapy (HBO) has historically been used to treat PAD patients. The underlying mechanism behind is through HIF-1a production and angiogenesis activation leasing to increased blood flow to the area. HBO has been shown to decrease circulating inflammatory markers and promote wound healing in CLI (Khan & Newton 2003).

Table 3.3 ***Advantages and disadvantages to current therapeutic approaches in peripheral arterial disease***

Therapeutic Approach	**Advantages**	**Disadvantages**
Supervised exercise-based therapy	• Non-interventional • Shown to improve walking distance	• Poor compliance
Pharmaceutical intervention	• Improve pain and quality of life in IC patients • Improves endovascular/surgical outcome	• May not be as cost effective as endovascular and surgical options
Endovascular intervention	• Short period of hospitalisation	• Survival rates may be lower long-term than open surgical options
Bypass	• Efficient with high survival rate	• Open surgery so comes with infection risk • Longer recover time than endovascular
Amputation	• Reduce immediate risk of sepsis and/or mortality	• Mentally/physiologically hard • Reduced mobility • Long recovery time
Compression/TENS devices	• Can be delivered at home by the patient • Readily available in pharmacies, online etc.	• Limited clinical evidence on effectiveness in relation to long-term wound

		healing and limb-outcomes.
Stem cell and gene-based therapies	• Some evidence to support improved CLI condition and reduction in amputation rate	• Expensive • Extensive techniques required to harvest stem cells and prepare gene therapy • Lack of clinical evidence

Current treatment options only aim to alleviate symptoms and impede disease progression. With treatment starting at simple modifications such as walking exercise and smoking cessation to then progressing to endovascular and surgical intervention, there remains a huge gap in treatment options between these two extremes. Currently, treatment begins at the later stages of PAD when severe pain and chronic ulcers have already ensued. Surgical and endovascular options can cause complications in elderly and frail patients as well as high rates of restenosis. Not all patients are eligible for revascularisation meaning their treatment options are extremely limited with long-term care often being required. Ultimately, in these cases, a lower limb amputation or palliative care is often required. There remains a strong need for alternative therapy in those who are refractory to conventional therapy.

3.1.5 PAD Outcomes

As discussed, current treatment options for PAD are limited. While these options do provide symptoms relief and many patients go on to have an improved quality of life for many years, PAD outcomes remain bleak. It is important to note that IC and CLI should not be grouped together when reporting outcomes as they undergo different treatment paths. Approximately a quarter of all patients originally presenting with claudication will develop more severe disease (Bauersachs et al 2019). IC patients rarely undergo major amputation with only around 3% undergoing the procedure over a 5-year period whereas 50% of CLI patients will undergo an amputation within the first year of diagnosis (Norgren et al 2007, Campia et al 2019).

The outcomes for CLI patients are the bleakest as the disease is very advanced and, in most cases, systemic. Around 40% of CLI patients have some form of amputation within 6 months of diagnosis (Uccioli et al 2018) meanwhile risk of major amputation at 1-year post-diagnosis is 50% (Campia et al 2019). After primary amputation, between 9 and 33% of CLI patients will die, this figure rises to between 26% and 82% 5 years post-amputation (Mastapha et al 2020). For those CLI patient who do not undergo intervention or intervention has failed, around 40% of these will lose a lower limb within 6 months and 20% will die within one year (Norgren et al 2007, Campia et al 2019). The overall mortality rate for CLI is 20% within 6 months of diagnosis which increases to 50% 5-years post-diagnosis (Uccioli et al 2018). The most common reasons for death in PAD sufferers are coronary artery disease and cerebrovascular disease, each contributing to 40-60% and 10-20% of deaths respectively (Campia et al 2019).

One recent study looked at post-intervention outcomes in 404 CLI patients (Rutherford 5 and 6). Patients who underwent endovascular intervention were followed up 30 days, 6 months and 12 months post-op. At 12 months, 78% of patients had not subsequently underwent an amputation and 71% of wounds had completely healed. However, rate of wound healing was less in patients classified in Rutherford 6 i.e. more severe disease. Only 10% of patients in this study went on to have a major amputation. Meanwhile, in patients who do not undergo any revascularisation intervention, approximately 22-70% underwent a major amputation at 12 months post-follow up. Those patients undergoing surgery exhibited a better 12-month survival rate compared to those who did not undergo revascularisation (Mustapha et al 2019).

Survival rates have improved with advances in medical interventions but there remains a need to improve the humanistic burden associated with quality of life and the social, financial and mental impacts of PAD on individuals.

3.1.6 Economic burden of PAD

The PAD population is large and consists mainly of elderly individuals. With an ever-aging population, the prevalence of PAD is on the rise. There is a significant economic burden

which comes with treating PAD which is likely to increase as the disease continues to prevail. The disease normally effects an individual for several years spanning from later in life and staying with them until death. As PAD cannot be cured, care, management and treatment are often required for the rest of an individual's life thus amounting to vast costs, for the individual or healthcare provider. Costs tend to amount due to dressing changes, hospitalisations, carer visits and surgery. The lengthy period of morbidity puts particular strain on health and social care resources with outpatient dressing and wound care management constituting the majority of care costs in patients with ulcers. Costs are even larger in patients with concurrent CAD due to higher rate of cardiovascular events and hospitalisation. Approximately 1000 per million new cases of CLI are diagnosed each year. In the UK, each lower limb amputation costs the NHS around £9440 with approximately 5000 major lower limb amputations performed yearly in the UK, this amounts to yearly costs totally over £50million (Ezeofor et al 2021). Data from the Oxford Vascular Study, a population-based incidence study, was analysed to assed long-term costs of CLI. Patients were recruited between 2002 and 2012 with a 5-year follow up to 2017. The mean cost of care over the five-year period was 46000 euros. Diabetic patients and those undergoing lower limb amputation cost around 12000 euros more than others (Luengo-Fernandez et al 2018). Expenses appear to be driven by hospitalization and amputation costs. A treatment which can be delivered at home, preventing the need for hospitalisation and amputation procedures may significantly reduce economic burden on healthcare systems.

3.2 Raynaud's Phenomenon

3.2.1 Definition of Raynaud's Phenomenon

In 1862, Maurice Raynaud was first to describe the phenomenon, which is now named after him, of vasospastic attacks and skin colour changes in the fingertips. Raynaud's initial experimentation was conducted in a group of 25 patients, 20 females, who exhibited digital colour changes upon cold exposure or under stress (Raynaud 1864). The phenomenon was not standardised until more recently and is now defined by vasospastic attacks leading to ischaemia and colour changes in the extremities.

Raynaud's phenomenon (RP) describes a cluster of symptoms rather than a disease entity. The symptoms typically arise as a result of vasospasm and reduction in blood flow upon exposure to cold temperatures though can also appear in response to emotional stress. RP can be either primary or secondary to another underlying condition. Secondary Raynaud's (SRP) appears due to an acquired disease which alters vascular reactivity. Underlying diseases with cause SRP are vast and include connective tissue disorders such as systemic sclerosis (SS). SS is a chronic connective tissue disease in which the immune system attacks the connective tissue causing vascular abnormalities in the skin and internal organs. In 90% of SS patients, RP is present (Belch et al 2017). Other conditions associated with SRP include hypothyroidism, Buerger's disease and frostbite sequelae. SRP can also arise as a result of occupational based hazards or a low body mass index and certain drugs. Primary Raynaud's Phenomenon (PRP) usually presents earlier in life, normally during teenage years to mid 20s, and is thought to be caused by a defective thermoregulatory system resulting in exaggerated responses to cold temperatures. Classic PRP effects both hands equally, with the thumbs being spared. Emotional stress appears to be a greater trigger in primary patients than secondary while cold exposure is an aggravating factor in almost all cases. In almost all (98%) of cases, the digits are affected however other body parts can also experience vasospastic attacks including toes, ears, nose, tongue, and nipples (Pauling et al 2019).

The prevalence of RP is estimated to be 5% in the general population however this varies within populations depending on local climates (Garner et al 2015). As high as 90% of all Raynaud's patients fall into the PRP category (Devgire & Hughes 2019). Young women are particularly effected and the prevalence of PRP can be as high as 30% in young women (Baines et al 2013). Approximately 90% of all RP cases are women with just 10% being male (Belch et al 2017).

3.2.2 Raynaud's Phenomenon Symptoms and Diagnosis

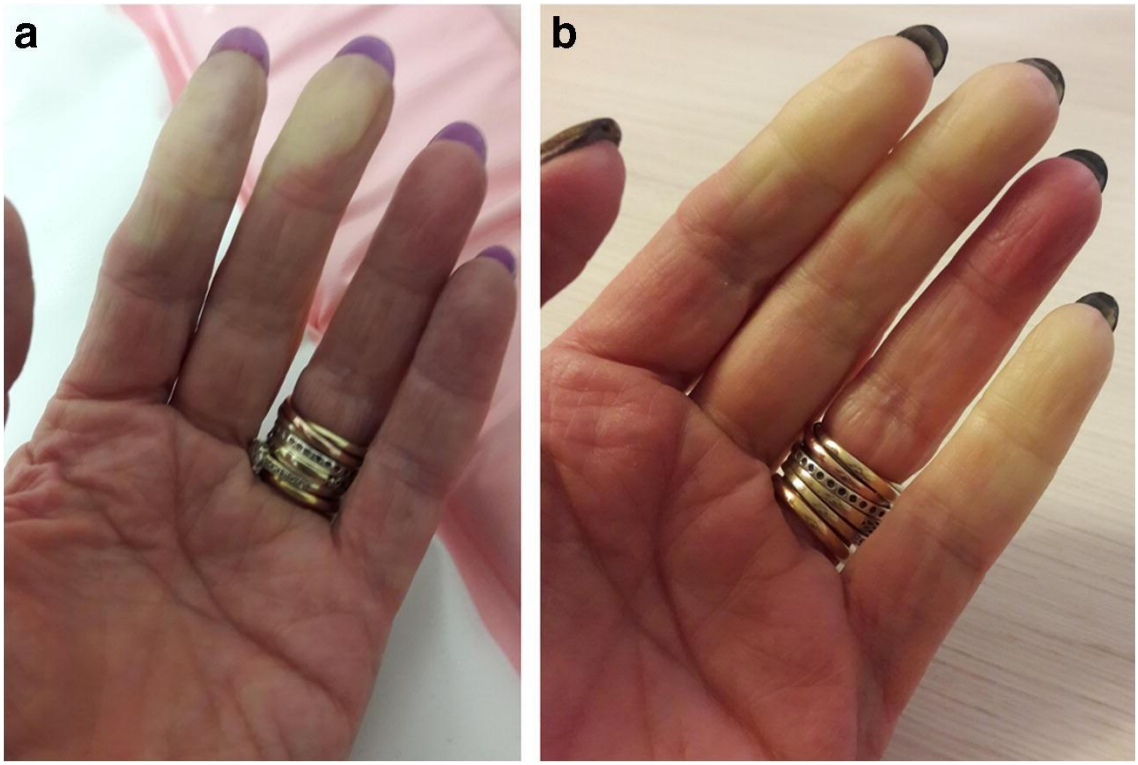

Figure 3.3 ***Raynaud's Phenomenon.*** Picture of a Raynaud's Phenomenon sufferer's hand during an ischaemic attack. During an attack, the digits undergo colour changes; pallor, cyanosis and rubor. The patient may also experience pain and swelling of the extremity. Reprinted from Clinical Rheumatology. **38** Pauling JD, Hughes M & Pope JE. Raynaud's Phenomenon - an update on diagnosis, classification, and management. Copyright (2019) with permission from Springer Nature.

Symptoms experienced during a vasospastic attack include discolouration, pain and swelling of the extremity which usually subsides. In most, but not all, cases there is a three-stage change in skin colour; pallor, cyanosis and finally rubor (Figure 3.3). Pallor or whitening occurs first due to the vasospasm and constriction of flow followed by cyanosis or blueing due to deoxygenation. Finally, rubor or redness appears alongside tingling upon reflow when the attack subsides. Not all three colour changes may be present in a RP case however for RP to be diagnosed, pallor must be present.

PRP usually develops independently of other underlying conditions and presents earlier in life than SRP, particularly in young women. As SRP develops due to an underlying condition, symptoms do not usually appear until later in life. Ischaemia in PRP is transient however in

SRP, ischaemia can be long-lasting and can therefore result in tissue damage and ulceration of the digits which are hard to heal and are prone to infection meaning some patients present with trophic changes.

The diagnosis of RP begins with a review of the medical history, symptoms and physical examination. Patients may be asked to keep a diary of attacks and take photos of the effected regions when an attack ensues in order to identify if pallor is present. Care must be taken to not confuse symptoms with other conditions which cause microvascular occlusion including occlusive vascular disease, hepatitis associated vasculitis, cryo diseases and hyperviscocity symptoms. A detailed medical history of the symptoms is taken. Whether digital pallor is present, symmetry, location of symptoms, age of onset and frequency of attacks are all noted. A full medical history will also help clinicians identify any underlying, secondary causes of the symptoms. Physical examination is carried out to identify any trophic changes, the possible presence of obstructive vascular disease or other associated disease. If a patient presents with RP symptoms later in life (>30yo), there is greater risk of developing connective tissue disease. Therefore, care must be taken in older patients, in their 30's and above, who present with RP symptoms and examination should be carried out to identify any vascular disease or autoimmune conditions. Peripheral blood pressures are taken to ensure no occlusive arterial disease is present. Approximately 60% of RP cases in patients over 60 years old is atherosclerotic in nature. When SRP is suspected, blood and urine tests are performed to test for underlying conditions and capillary microscopy is commonly used to identify abnormalities in the nailfold vessels. Any signs of SRP leads to referral to secondary care.

Nailfold capillaroscopy is a non-invasive technique used to visualise the microcirculation and in particular the capillaries of the nailfold, which are orientated parallel to the surface of the skin (Figure 3.4). The technique allows clinicians differentiate primary from SRP. Nailfold capillaroscopy has been around for many years. The presence of abnormal nailfold capillaries has been known to be predictive of underlying connective tissue disease and systemic sclerosis for the past 40 years however it is only within the last two decades that nailfold capillaroscopy has become a predictive tool used clinically. The technique visualises the peripheral capillaries and analyses microvascular abnormalities which may be present in those exhibiting SR. Morphological abnormalities in the microvessels are present in

connective tissue disease (CTD). These changes include changes in capillary size, density and alterations in the normal capillary loops. A variety of devices can be used to perform nailfold capillaroscopy such as light microscope, dermatoscope and high magnification video capillaroscopy.

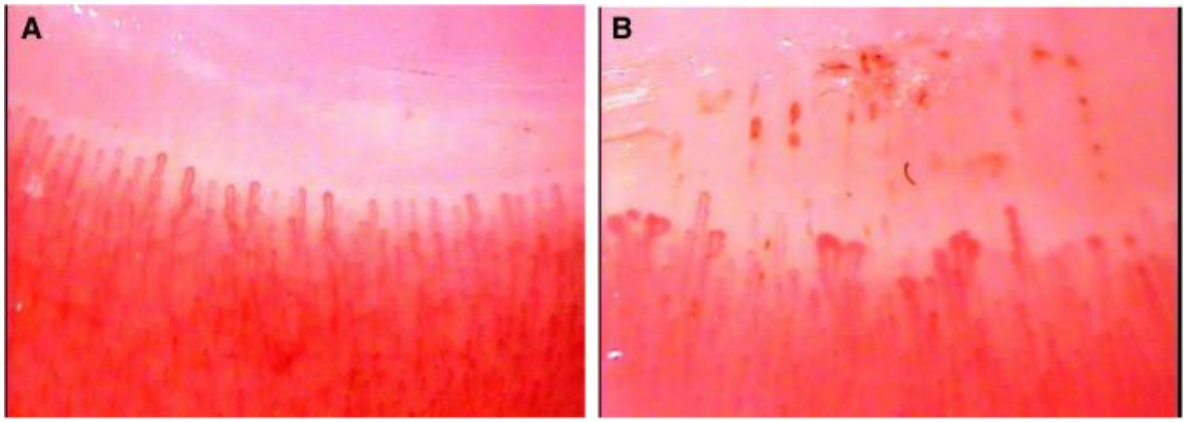

Figure 3.4 ***Nailfold capillaroscopy.*** Nailfold capillaroscopy is a common technique used to visualise the nailfold microcirculation. Visualisation of the microcirculation at this location allows clinicians to determine if Raynaud's Phenomenon is primary or secondary as secondary disease usually exhibits morphological abnormalities in the vessels including abnormal capillary size and density as seen here (B versus healthy A) (Reprinted from Kayser et al 2019 under the terms of the Creative Commons Attribution 4.0 International License).

Infrared thermography is used to measure skin temperature alongside a temperature challenge. In patients with PRP, there is a delayed rewarming response after cold exposure. In SRP, particularly those with underlying systemic sclerosis, rewarming is not present. This technique is not routinely used outside of specialist RP centres as the equipment needed is very expensive.

3.2.3 Pathogenesis of Raynaud's Phenomenon

The pathogenesis of RP is complex and not fully understood to this day although a mound of experimental work has been conducted to investigate various causes of RP including alterations in the control of vascular tone and thermoregulation. It is likely that PRP occurs as a result of functional abnormalities however SRP is thought to arise due to structural changes in the microvessels and digital arteries which occur as a result of underlying conditions such as SS.

In PRP, there is thought to a functional alteration in the balance of thermoregulation. In normal physiology, upon cold exposure, heat loss is reduced by an increase in sympathetic tone and locally enhanced vasoconstriction involving alpha-2 adrenergic receptors (A2AR). A2AR are G protein-coupled receptors found on vascular smooth muscle cells which are activated by noradrenaline. A2AR mediates cold-induced vasoconstriction and has been shown to be more abundant in the smooth muscle cell membrane at cooler temperatures (Jeyaraj et al 2001, Eid et al 2008). Greater expression of A2ARs on the cell membrane leads to increased sympathetic vasoconstriction. PRP patients have hyperactivity of the sympathetic nervous system, resulting in overactivation of A2ARs. Upon cold exposure, A2AR are upregulated in smooth muscle, contributing to enhanced vasoconstriction in response to noradrenaline which is released under central control. Vasospasm can be prevented when A2AR antagonists are administered (McNeill et al 1999).

Sex hormones, in particular oestrogen, may also play a role in overactivation of ARARs which may explain the high incidence of PRP in females (Serizawa et al 2017). PRP is more common in premenopausal women and cold sensitivity is heightened at certain points of the menstrual cycle (Greenstein et al 1996). Oestrogen is known to interact with central and peripheral thermoreceptors. At approximately halfway through the menstrual cycle, oestrogen levels are at their highest. During this time, it has been demonstrated that noradrenaline-mediated vasoconstriction is also at its highest (Chan et al 2001). Male rats incubated with oestrogen exhibit increased A2AR expression and increased vasoconstriction upon cooling suggesting oestrogen is involved in the increased activity of A2AR vasoconstriction (Eid et al 2007).

As described, the underlying cause of PRP is functional abnormalities. The pathophysiology of SRP can be more complex with underlying abnormalities being vascular, neural or intravascular resulting in digital cutaneous vascular injury and structural changes leading to abnormal reactivity. Structural abnormalities can include changes to the endothelium such as endothelial cell apoptosis and expression of adhesion molecules which may result in intimal thickening and vessel occlusion resulting in reduced blood supply, similar to that seen in atherosclerosis. Like in other diseases, the endothelium can become damaged due to underlying conditions thus causing excessive vasoconstriction. Ischaemia-reperfusion injury

may occur in RP patients, particularly those with SRP, leading to oxidative stress which contributes to endothelial dysfunction.

While endothelial function is likely to be compromised in SRP, it was previously thought it be unaffected in PRP. However, evidence has suggested endothelial reactivity may be affected in PRP. Studies using laser Doppler imaging alongside iontophoresis and ultrasound have assessed endothelium-dependent and independent vasodilation of small and large vessels, respectively. A reduction in endothelial-dependent vasodilation has been demonstrated in patients with systemic sclerosis as well as PRP and even an impairment in both endothelium-independent and dependent vasodilation of the micro vessels has been demonstrated using the iontophoresis method (Matucci-Cerinic et al 1990, Lekakis et al 1998, Freedman et al 1999, Khan et al 1999, Herrick 2000, Anderson et al 2003, Anderson et al 2004, Bedarida et al 1993, Smith et al 1999, Khan et al 1997). A study including 40 patients with RP (20 PRP, 20 SRP) demonstrated higher circulating levels of asymmetrical dimethylarginine, an inhibitor of eNOS. Furthermore, circulating endothelin-1 levels were increased in those with SRP (Rajagopalan et al 2000). Interestingly, Local NO delivery increases blood flow in RP patients (Tucker et al 1999). Prostacyclin is another vasodilator released by the endothelium. A study on rat aortic rings demonstrated that upon cold exposure, production of prostacyclin is impaired however another study provided evidence that patients with secondary RP have higher circulating levels of prostacyclin and therefore may be prostacyclin resistant (Jeremy et al 1988, Belch et al 1985). Abnormalities in the endothelium are likely to occur early and can result in intimal thickening leading to occlusion of the vessel as seen in patients with systemic sclerosis.

Genetics is likely to play a part in the development of RP as approximately half of all sufferers have a first-degree relative with the condition (Maundrell and Proudman 2015). Cigarette smoking is commonly associated with cardiovascular disease, including vascular diseases such as RP. Smoking can induce ischaemic attacks and increase their frequency with the link most likely being endothelial dysfunction and oxidative stress. Other aggravating factors may include caffeine.

3.2.4 Current Treatment and Management Options for Raynaud's Phenomenon

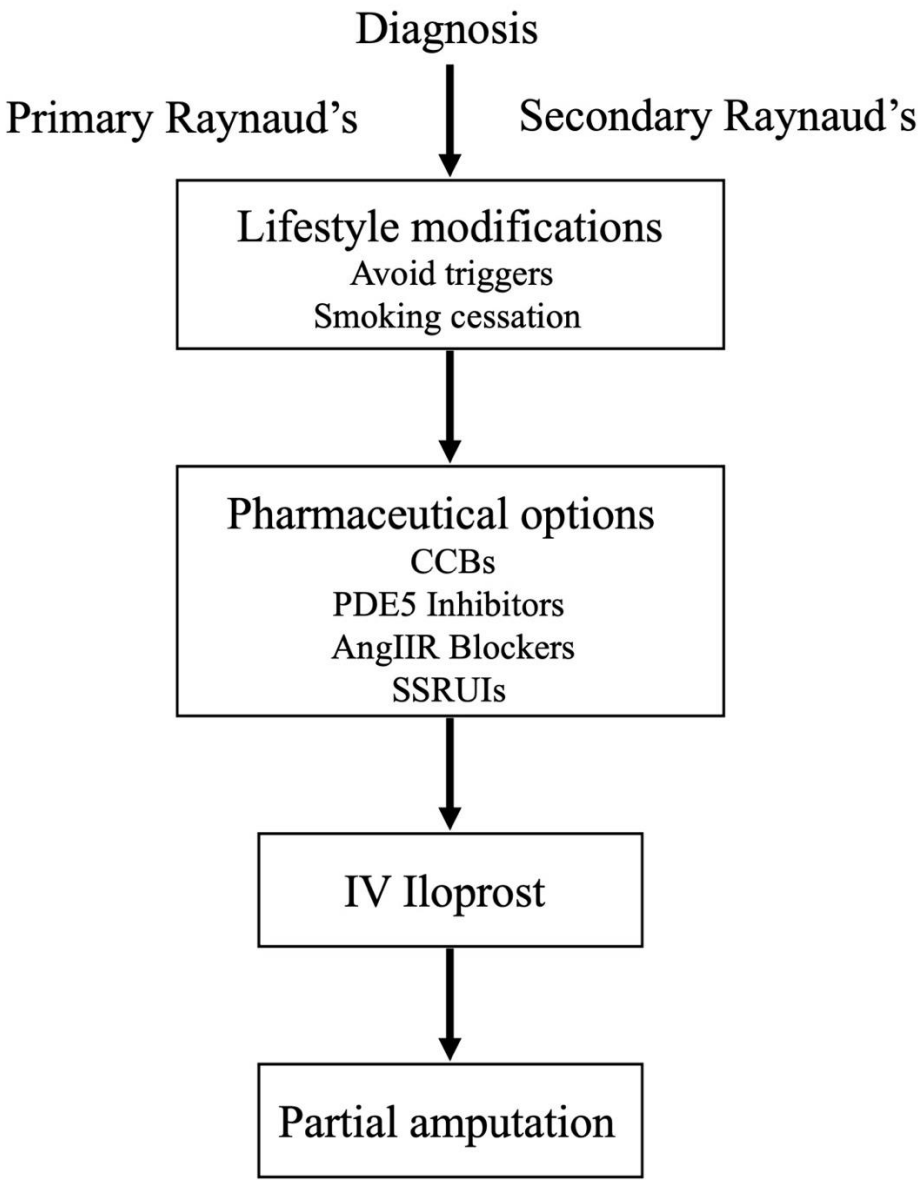

Figure 3.5. ***Treatment path for Raynaud's Phenomenon.*** Upon diagnosis of RP, treatment options depend on whether primary or secondary RP was diagnosed, severity of disease and impact on daily life. First line options include lifestyle modifications such as avoiding ischaemic attack triggers and smoking cessation. If these are not successful, a number of pharmaceutical options are available including CCBs, PDE5 inhibitors, angII receptor blockers and SSRUIs. In more severe cases where medication is not effective and trophic changes are present, IV iloprost can be administered to induce vasodilation. In RP patients who are not responsive to all other treatment options and/or develop ischaemic tissue, partial amputation of the digits may be considered. RP = Raynaud's phenomenon, CCB = calcium channel blockers, PDE5 = phosphodiesterase 5, AngII = angiotensin II, SSRUI = selective serotonin reuptake inhibitor, IV = intravenous.

After diagnosis of RP and further examination to determine any secondary causes, the patient will be classified as having either primary or secondary RP which will determine the following treatment route (Figure 3.5). Management of RP is highly dependent on the severity. Those with mild RP who experience infrequent attacks, can usually continue their normal daily lives with slight lifestyle modifications such as avoiding triggers and smoking cessation. However, in those who experience regular, painful attacks and especially in those experiencing trophic changes such as ulceration, treatment is required. The effect of pain from vasospastic attacks on an individual's daily life is sometimes undermined. In a 15-country wide online survey study of 443 self-reported RP patients, almost 80% of respondents said they had to make at least one modification to their life due to their symptoms (Hughes et al 2015).

Treatment aims to decrease attack frequency and severity. Lifestyle and pharmaceutical options are often utilised with the aim of inhibiting excessive vasoconstriction, enhancing vasodilation, reducing endothelial dysfunction, and attenuating platelet aggregation. Lifestyle changes are recommended in the first instance such as avoiding cold temperatures and smoking cessation, as these can exacerbate the symptoms. If lifestyle modification proves ineffective, pharmacological intervention is considered.

Drug therapy is considered in individuals whose symptoms interfere with their daily life and are not relieved by lifestyle modifications. Vasodilator drugs are used with the aim of increasing blood flow to the affected area. Calcium channel blockers (CCB) such as nifedipine are the first line of treatment for RP and are commonly used to manage ischaemic attacks associated with RP through blood muscle relaxation and vasodilation resulting in increased blood flow. With drug treatments, patients are initially prescribed a low dose which is gradually increased depending on effectiveness and patient response to dose-limiting side effects include dizziness, nausea, headache, and palpitations. CCB use should be avoided in those with hypotension, angina, myocardial infarction, hypertrophic cardiomyopathy, and severe pulmonary hypertension. If nifedipine is not tolerated, then other CCBs can be prescribed including amlodipine and lercanidipine. The effectiveness of CCBs is debated. A recent review of 296 patients from several clinical trials found only a small effect of CCBs on the number of attacks in PR versus placebo (Ennis et al 2016). It is worthy nothing that some of the studies included in the review were small scale and data quality between trails

was variable. A recent Cochrane meta-analysis of 39 randomised-controlled trials revealed treatment with CCBs reduces the severity of attacks as well as frequency by one third and that higher doses, if tolerated, are likely to be more effective. Results also indicated that PRP sufferers are more responsive to CCBs that secondary patients (Rirash et al 2017).

If CCBs are not efficacious, other vasodilator drugs are considered including phosphodiesterase-5 (PDE5) inhibitors, angiotensin receptor blockers, selective serotonin-re-uptake inhibitors, alpha-blockers, and angiotensin-converting enzyme inhibitors. In those with sRP, Phosphodiesterase-5 (PDE5) inhibitors such as sildenafil are increasingly being prescribed with several trials suggesting a significant benefit however these trials have been short-term testing and the long-term benefits are unknown (Shenoy et al 2010, Herrick et al 2011, Caglayan et al 2012, Lee et al 2014). However, the use of these alternatives in RP remains controversial and there remains a need for these alternative vasodilators to be tested in large placebo-controlled trials (Stewart and Morling 2012).

Some patients try vitamin supplementation, alongside standard therapy, such as ginkgo biloba, vitamin C and vitamin E, while there is little evidence supporting the use of vitamin supplements in RP, the overall risk is low so is not routinely advised against.

Severe RP, particularly those suffering from systemic sclerosis, can lead to digital ulceration. In this case, intravenous prostanoid therapy with iloprost can be utilised. Although IV iloprost has proven to be beneficial in SRP patients, care is required to monitor vasodilator side effects on blood pressure which can result in hypotension (Herrick and Muir 2015). Therefore, iloprost treatment is reserved for severe SR patients with digital ulceration. Surgical intervention is considered in those with refractory SRP where pharmaceutical management has failed. Surgery is not considered in PR patients. Most often, partial digital amputation or ischaemic tissue removal is performed. In the case of critical digital ischaemia, full finger amputation may be required. In a large cohort study of systemic sclerosis patients, 7.4% suffered from serious digital complications including ulcerations or digital ischaemia within an 18-month period. Critical digital ischaemia developed in 1.6% of patients and 1.4% developed digital gangrene (Nihtyanova et al 2008).

Chapter 4: Literature Review of Negative Pressure

4.1 Historical overview of negative pressure in medicine

Historically, ‘cupping’ was one of the first pressure-altering methods used in medicine, dating back to 700BC, with the aim of partly withdrawing blood from circulation. In the 1800s, Junod utilised a ‘cupping’ method to create a vacuum using a device which could reversibly withdraw a large quantity of blood from systemic circulation. The device was intended to be used in the treatment of inflammation and fever and was additionally applied to induce syncope during surgery (Junod 1834, Junod 1838) (Figure 4.1A). Following this, Edgar Bluck created a novel method of increasing limb blood circulation by applying an alternating negative pressure of -80mmHg and positive pressure of +80mmHg (Bluck 1888). In the 1900s, Bier proposed the idea that hyperaemia could be used to treat disease with the theory being diseased areas of the body have naturally higher blood flow and therefore artificially increasing blood flow to the affected area would promote healing. This was one of the earliest descriptions of pressure being utilised in ulcer healing (Bier 1905). An early description of negative pressure being used in the treatment of peripheral vascular disease came in 1917 when Sinkowitz utilised Bier’s hyperaemia technique to produce a constant negative pressure on the foot of four Buerger’s patients resulting in improved peripheral circulation indicated by increased skin temperature as well as reduced pain and wound healing (Sinkowitz & Gottlieb 1917).

Developments from ‘cupping’ came around 50 years later in the form of lower body negative pressure (LBNP). LBNP is a research technique involving a patient lying down inside a large pressure chamber which is normally sealed around the iliac crests with negative pressure produced inside the chamber using a vacuum pump. The first reference to the technique was in the 1950s when Restall and Smirk demonstrated that LBNP applied to the lower limbs of individuals resulted in reduced arterial pressure (Restall & Smirk 1952). LBNP has since been utilised as an important non-invasive research tool as a means of reducing central blood volume and to create blood pooling in the periphery. In space flight where there is an absence of gravity, astronauts use LBNP devices daily to aid in blood circulation to the periphery prevent orthostatic complications. Research applications include investigating orthostatic tolerance, responses to space flight, assessment of autonomic function and baroreceptor function and assessment of cardiovascular response to exercise (Stevens & Lamb 1965). LBNP has several effects on the cardiovascular system with responses varying depending on

magnitude of pressure applied. Low magnitude LBNP (< -20mmHg) offloads cardiopulmonary baroreceptors leading to sympathetic nervous system activation, peripheral vasoconstriction and stabilisation of arterial blood pressure. There is no change in heart rate at this level of negative pressure (Zoller et al 1972). High magnitude LBNP (> -20mmHg) not only elicits cardiopulmonary but also arterial baroreceptor offloading causing a decrease in stroke volume. As stroke volume decreases, cardiac output also decreases eventually eliciting an increase in heart rate (Furlan et al 2001). Mean arterial blood pressure remains relatively constant due to peripheral vasoconstriction causing increased systemic resistance. Furthermore, LBNP elicits a change in transmural pressure. The change in blood pressure elicited by LBNP causes vasodilation through endothelial activation, and a consequential increase in blood flow (Greenfield & Patterson 1954, Coles & Greenfield, 1956). Although there is strong evidence to suggest arterial baroreceptors are unaffected by low magnitude LBNP, contradicting evidence has suggested both cardiopulmonary and arterial receptor offloading during low magnitude LBNP, with a change in MAP being recorded however this evidence is limited (Hisdal et al 2001).

Although the use of altering pressure with vacuum systems has been around for centuries, the method of application has varied, producing varied results. In the past, some methods have applied negative pressure continuously. Several studies have subsequently demonstrated that the continuous application of negative pressure is counter-productive and leads to decreased blood flow to the area. Skagen and Henriksen demonstrated that local application of a constant negative pressure of -20mmHg to the arm for as little as two minutes causes vasoconstriction by increasing vascular resistance and decreasing peripheral blood flow. When the magnitude of negative pressure is increased to -40mmHg there was a further increase in resistance and reduction in flow signifying that as the magnitude of negative pressure increases, the fall in blood flow is greater as continuous pressure is applied (Skagen & Henriksen 1983). Likewise, Stranden demonstrated that application of continuous negative pressure for one minute to the lower body of healthy participants halved femoral blood flow (Stranden 1984). This response was abolished with local neural blockage using lidocaine suggesting vasoconstriction is due to a local sympathetic axon reflex (Skagen & Henriksen 1983). The venoarteriolar reflex is a local sympathetic response to a change in transmural pressure which can occur when a limb changes from a lying to standing position and reduces blood flow to the limb. The role of this physiological response is to constrict arteries when

veins over distend in order to control fluid balance and oedema. Therefore, negative pressure with brief oscillations, or intermittent negative pressure (INP), is believed to circumvent this local sympathetic reflex and allow for prolonged increases in blood flow. Based on the idea that negative pressure increases blood flow, at least in the short-term, intermittent negative pressure (INP) has been utilised to increase blood flow. INP with brief oscillations of negative pressure can circumvent the venoarteriolar reflex and increase peripheral blood flow.

Early uses of intermittent negative pressure (INP) date back to the 1930's when Herrmann & Reid utilised INP in a technique named passive vascular exercises (PAVAEX) which delivered a negative pressure of -80mmHg in cycles of 10 seconds negative pressure and 5 positive pressure to treat vascular diseases (Figure 4.1B). Long-term application of PAVAEX to patients with lower limb arterial disease resulted in granulation tissue formation on wounds and an increased skin temperature indicating increased flow to the area (Skagen & Henriksen 1983, Stranden 1984). Similarly, Landis & Gibbon developed a similar technique with a negative pressure of -120mmHg being applied to treat arterial disease in the lower limbs (Landis & Gibbon 1933). In the 1990s, a Danish group produced a device, Vacusac, which applies negative pressure in 5-10s oscillations, aimed at treating patients with a range of vascular diseases as well as sports injuries. In a randomised-controlled trial, 22 patients with intermittent claudication were assigned treatment with an active or placebo device. After 2 months, patients using the active device recorded increased maximal walking distances as well as a positive change in ankle-brachial pressure index (ABPI), a marker of atherosclerotic lower limb arterial disease (Himmelstrup et al 1991). However, use of the device required the patient to undress and 'wear' the bag-like-device while supine and operation to be carried out by a healthcare professional, deeming it unpractical for home use.

Although early negative pressure delivery methods seemed effective and produced good clinical outcomes for vascular patients, there were several draw backs. It was not possible for patients to administer the treatment themselves as devices were heavy, expensive and required a clinician to operate them throughout delivery. Furthermore, the only data on the effects of treatment were broadly clinical markers and the mode of action remained unclear.

In 1969, Smyth provided evidence of the effects of INP on blood flow velocity. An ultrasound device was used to measure femoral flow velocity in study participants who administered INP of -150mmHg twice weekly for 6 weeks. Results showed the same clinical benefits of previous studies; increased walking distance and wound healing as well as a 292% increase in resting peripheral blood flow versus pre-treatment. Furthermore, during application of negative pressure an increase in femoral flow velocity was observed (Smyth 1969). Although this study provided strong evidence on the relationship between INP treatment and blood flow velocity, the data focussed on macrovascular blood flow and did not provide information regarding the microcirculation. Structural and functional damage to the microcirculation is a precursor to atherosclerotic diseases, including peripheral vascular disease, and is therefore an important component to assess when investigating methods of treating such disease.

While negative pressure has been used extensively as a treatment modality for patients with vascular disease of the lower limbs, a small amount of research has investigated the possible benefit in vascular diseases elsewhere in the body. In the 1930s, Braeucker successfully utilised Bier's hyperaemia technique in the treatment of 19 RP patients (Braeucker 1931). Gill and Walder later replicated Smyth's method of INP application in a group of 7 RP patients. After 4 weeks of 30 minutes INP 3 times a week, patients exhibited increased finger blood flow as measured by venous occlusion plethysmography. Furthermore, skin temperature of the forearm significantly increased throughout treatment and was sustained post-treatment. No changes were recorded in 5 control patients thus demonstrating the applicability of negative pressure in a further patient group (Gill & Walder 1974). In both of these studies, it is unclear whether participants exhibited primary or secondary RP.

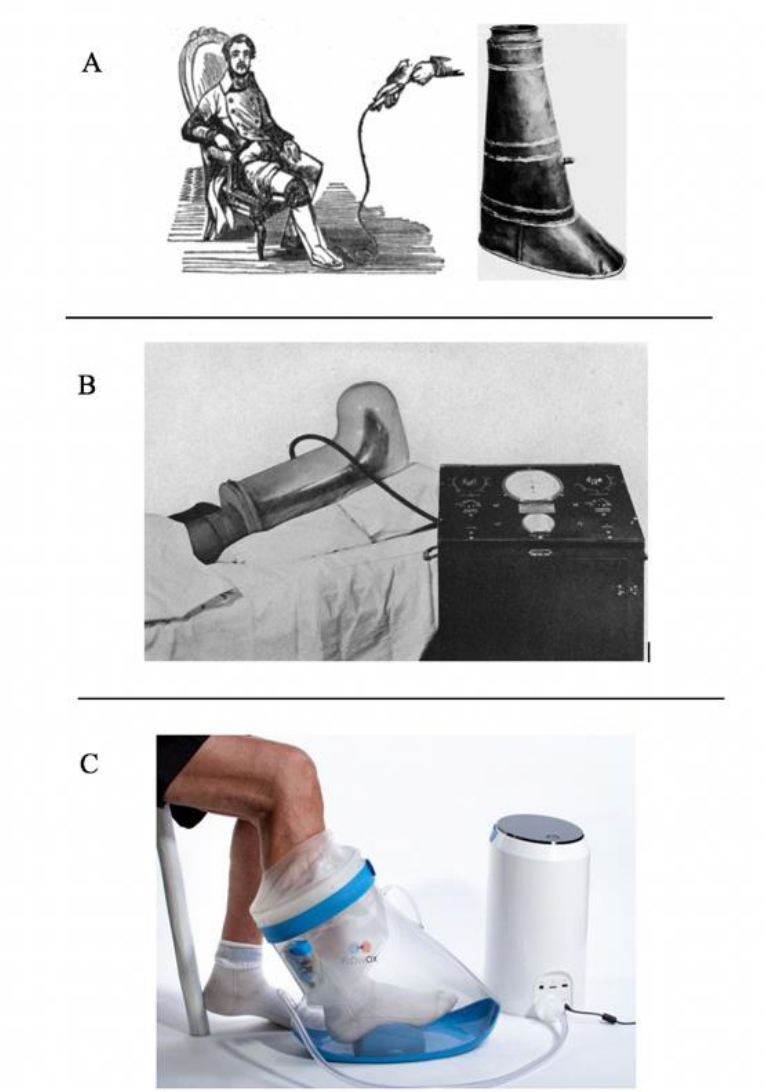

Figure 4.1 ***Historical and current negative pressure application devices.*** (A) Junod's boot. The metal device designed by Junod was shaped like a boot to accommodate a single leg. A pump was used to draw air out of the air-tight boot, creating a vacuum (Crystal & Salem 2015) (Reprinted from Journal of Anaesthesia History. 1(2) GJ Crystal & MR Salem. Lower Body Negative Pressure: Historical Perspective, Research Findings, and Clinical Applications. 49-54. Copyright (2015) with permission from Elsevier). (B) PAVAEX (passive vascular exercise) treatment device. The system comprised of a glass treatment boot and treatment unit (right). During treatment, the lower limb was inserted into the boot and elevated above the body (Herrmann & Reid 1934). (C) Flow-Ox Device. The Flow-Ox system consists of a plastic pressure chamber and control unit. The pressure chamber comfortably accommodates the leg with inflatable padding to stabilise the calf and a rubber seal to ensure an air-tight environment. The control unit monitors user compliance and controls pressure within the pressure chamber. Users are asked to be comfortably seated with one leg in the pressure chamber. The INP protocol begins when the base is turned on. A timer is displayed on the control unit so patients can track their usage. If there is an air leak from the pressure chamber, an error message will alert the user to adjust the padding and/or seal.

4.2 Current applications of negative pressure

As described, negative pressure and intermittent negative pressure have been extensively investigated as a treatment modality for many decades with successful application in a variety of vascular patient groups, particularly PAD. Currently, a number of treatment modalities which utilise pressure are routinely offered by healthcare systems and are widely available including pneumatic compression devices and negative pressure wound therapy. Recently, further work has been done to optimise and prove the effectiveness of intermittent negative pressure in PAD. Here we discuss current pressure-based treatments widely available then go on to explore more recently advances in pressure therapy research.

Pneumatic compression devices, as described previously in section 3.1.4, are readily available for patients to purchase from pharmacies and supermarkets however are not routinely offered by clinicians due to lack of clinical evidence regarding their benefits. The physiological mechanism behind intermittent pneumatic compression is thought to involve shear stress and the endothelium. At the site of compression, the pressure gradient increases and accelerates the blood forward thus facilitates venous emptying. There is improved venous emptying and decreased venous pressure leading to an increase in the arterio-venous pressure gradient eliciting arterial inflow. Distension and increased flow exert strain and shear on the arterial endothelium, activating it to produce NO which promotes vasodilation. In a study of intermittent pneumatic compression application in rats, arterial and venous vasodilation was observed with maximum vasodilator response being achieved after 30 minutes of application. This vasodilator response was inhibited by an NO inhibitor therefore provided strong evidence that the endothelium and NO play an essential role in the vasodilator effects of pneumatic compression (Liu et al 2005). High-pressure intermittent leg compression (HPILC) is another alternative treatment for patients with claudication which utilises intermittent pneumatic compression exerting pressures >100mHg. Pneumatic compression has been demonstrated to significantly increase popliteal flow rate (Bemmelen et al 1994). A systematic review and meta-analysis of 8 randomised controlled trails evaluating HPILC efficacy in IC revealed the treatment modality is capable of eliciting an significant increase in absolute claudication distance, compared to controls (Oresanya et al 2018).

More commonly used clinically, negative pressure wound therapy (NPWT) is a non-invasive treatment option for chronic wounds. NPWT, which is delivered via wound dressings, applies a high magnitude intermittent or continuous negative pressure of up to -125mmHg directly to a wound area in order to remove excess fluid and promote healing. Although NPWT is routinely used as an effective treatment method, dressings require regular changing, up to three times a week by a healthcare professional. Moreover, unlike data from studies applying negative pressure to larger areas, which demonstrate an increased blood flow can be achieved, studies have demonstrated NPWT decreases local blood flow around the area being treated therefore can be considered as an effective wound treatment but does not address the underlying ischaemic condition and lack of blood flow to the limb (Borgquist et al 2010).

In 2010, a Norwegian group developed Flow-Ox, a negative pressure device which applies INP to the calf and foot. The cyclical INP protocol comprises of 10 seconds -40mmHg followed by 7 seconds atmospheric pressure (Figure 4.1C). The device aims to promote blood flow to the affected limb of PAD sufferers, reducing claudication pain and aiding in chronic wound healing. One advantage to this device, which was not present for previous devices, is patients can deliver the treatment themselves in their homes thereby decreasing burden on the healthcare system. The use of -40mmHg was justified in a recent study which evaluated different magnitudes of negative pressure to be used in INP therapy ranging from 0 to -60mmHg on blood flow responses in PAD patients as measured by ultrasound and laser Doppler methods. Optimal increases in blood flow were achieved by administering -40mmHg in cycles alongside atmospheric pressure. This pressure magnitude was the lowest magnitude at which significant changes in blood flow relative to baseline were observed (Hoel et al 2019).

Early studies using Flow-Ox focused on the effects in healthy volunteers with a single application. Application of INP for two minutes increased arterial blood flow velocity by 44% and blood flow in the skin, as measured by ultrasound and laser Doppler methods, indicating increased blood flow to both the macro- and micro-circulation. These results were shown to be specifically INP-induced as application of constant negative pressure without oscillations decreased blood velocity in the arteries of the foot (Sundby et al 2016a).

Sundby went on to demonstrate a similar effect in a group of 20 PAD patients. Using Flow-Ox, INP was applied to the foot for 10 minutes resulting in a 46% improvement in local arterial blood flow velocity as well as increased skin blood flow, similar to that seen in healthy subjects. Interestingly, the rise in blood flow observed was sustained for five minutes post-INP suggesting improvement is prolonged (Sundby et al 2017). Sundby further explored the longer-term effects of repeated INP application in patients with lower limb ischaemia. Four patients who suffered with lower limb chronic wounds were recruited and received INP treatment for two hours per day for 8 weeks, administered themselves, in their own homes. Similar to studies described earlier, all patients exhibited either partial or complete wound healing suggesting long-term improved blood flow to the area (Sundby et al 2016b). A recent study was the first randomised sham-controlled trial to evaluate the effects of long-term INP treatment on walking distance in patients with intermittent claudication. The study of 72 patients found pain-free walking significantly increased after 12 weeks of daily treatment versus those who received a sham device however maximal walking distance was found to no have significantly increased (Hoel et al 2021a). 10 of the original patients who received the active device continued to apply INP daily for a further 12 weeks. After 24-weeks, significant improvements in pain-free and maximal walking distance, compared to baseline. No significant changes in ABPI were recorded for either groups at either 12-week or 24-week follow up (Hoel et al 2021b). Furthermore, while LBNP has been shown to elicit cardiovascular responses, acute application of INP has minimal effects on central haemodynamics with negligible changes in heart rate and mean arterial blood pressure being reported in these studies of healthy and diseased subjects (Sundby et al 2016a, Sundby et al 2017). This is important due to many PAD patients exhibiting concomitant coronary and/or cerebrovascular disease where changes in central circulation may elicit cardiovascular events.

With regards to the systemic effects of negative pressure on blood borne inflammatory and oxidative stress markers, a recent placebo-controlled trial evaluated the effects of 12 weeks INP therapy on biomarker levels in IC patients. Biomarkers tested included VCAM-1, ICAM-1, von Willebrand factor (vWF), L-arginine, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA). After 12 weeks of daily INP treatment, no significant differences in any biomarkers were recorded between the active and placebo groups. However, a significant reduction in vWF levels was detected between the two time

points in the treatment group but not the placebo group. This marker is a glycoprotein stored in endothelial cells and serves functions in platelet adhesion during endothelial injury therefore a reduction in its circulating levels may suggest improvement in endothelial injury with INP treatment (Hoel et al 2021c).

Although providing clear evidence INP application brings about changes in wound healing and blood flow, these studies were relatively small scale both in the number of participants and length of study. A larger scale clinical trial is required to provide strong evidence of the use of Flow-Ox as a treatment modality in patients with peripheral arterial disease and exploration into patients with more advanced disease is warranted. Furthermore, these patients exhibit differing severities of the disease and whether Flow-Ox is beneficial in one or all of these stages of severity requires investigation as an effective treatment modality should be beneficial to the majority of treatable patients.

Some studies have suggested intermittent pressure may not bring about clinical changes in PAD patients. Vacumed, a German intermittent vacuum therapy (IVT) device, was tested in IC patients to assess the effects of intermittent pressure on walking capacity. 48 patients were randomised to either undergo lifestyle modifications alongside IVT treatment or lifestyle modifications only. After 6 weeks of treatment, ABPI decreased in both groups with no significant differences between the two groups. Walking distance and time to pain decreased in the control group however did not improve with IVT intervention. They concluded that IVT therapy with Vacumed had no significant impact on distal blood pressures or walking capacity in IC patients (Afzelius et al 2018).

A similar placebo-controlled study assessed the effects of IVT alongside supervised exercise therapy (SET) in 70 IC patients. After 6 weeks of treatment, no significant changes in maximum or functional treadmill walking distance or 6MWT were recorded in the group receiving IVT therapy, compared to those who received a sham device. Quality of life improved equally in both groups (Hageman et al 2020).

Therefore, while studies with the Flow-Ox device method of intermittent pressure delivery have proved successful in improving walking distance in PAD patients, other methods of intermittent negative pressure delivery have not been as successful. This may be due to differences in treatment length, time, delivery and magnitude of pressure applied.

Much research efforts and clinical applications of INP have focused on application to the lower limb for treatment of arterial diseases however a recent study conducted experimental work on the upper limb to assess the impact of INP on endothelial function. Shear rate fluctuations were induced by application of INP to assess the effect on macrovascular endothelial function which as measured by brachial flow mediated dilation (FMD). Using a cylindrical device, INP was applied to the forearm of 15 healthy men for 30 minutes. The other arm was unexposed to INP and acted as a control. FMD was tested bilaterally at the brachial artery. Data demonstrated a significant increase in brachial FMD (+2%) compared to baseline in the experimental arm but not the control arm (Holder et al 2019). This suggests, in the upper limb, INP can induce an improved ability of the artery to dilate in response to blood flow, providing initial evidence of a possible physiological mechanism behind INP however further experimental work is warranted to determine if these results translate to the lower limb. Moreover, this work was conducted in the macrocirculation and whether the same is true for the microcirculation, whose function is heavily impacted by atherosclerosis, requires investigation.

4.3 Potential mechanisms

Thus far, research surrounding INP has focussed on demonstrating it as a treatment modality for vascular diseases. While the data has provided strong evidence that this is possible, there is little to no literature describing the mechanisms behind the blood flow changes elicited by INP. It is essential the mode of action is fully understood to allow the use of INP to be justified and applied correctly. Herein, potential mechanisms will be explored however, as the literature is limited, these are partly speculative based on the data and literature to date.

4.3.1 Flow Model

Based on the information stated in Chapter 1 regarding Hagen-Poiseuille's equation and the effect of vessel radius on resistance and flow, it can be theorised that upon application of negative pressure, there is a decrease in venous pressure. Indeed, upon application of negative pressure, Smyth observed pressure inside the veins as being similar to the magnitude of pressure applied (Smyth 1969). A decrease in venous pressure produces a greater arterial inflow due to an increase in the pressure difference between veins and arteries. A few seconds

of constant negative pressure elevates vascular resistance and reduces the pressure gradient between arteries and veins resulting in blood flow declining toward baseline. Furthermore, changes in transmural pressure, have been observed upon exposure to negative pressure. As described, positive external pressure decreases transmural pressure (Raju et al 2016). Therefore, it can be theorised that when negative pressure is applied there is an increase in transmural pressure, creating a greater difference in pressure across the blood vessel wall, leading to vasodilation and improved flow (Greenfield & Patterson 1954).

According to Poiseuille's equation, if the vessel radius is reduced, due to stenosis, resistance in that segment increases. Turbulent flow, as seen in branch points of the arterial system and areas with stenosis, increases the perfusion pressure required to maintain flow. Stenosis increases resistance in the effected vascular segment. An increase in resistance results in reduced distal perfusion pressure and blood flow. According to the equation, if the vessel radius reduces by a half, there theoretically will be a 16-fold increase in resistance causing a 16-fold decrease in blood flow assuming the vessel is isolated. However, the major limb arteries are not isolated and are in series with other resistance vessels therefore a stenosis does not have such a significant haemodynamic effect. The vessel radius has to be reduced by 60% or more to elicit a significant haemodynamic effect. Collateralisation occurs in the circulation distal to stenosis resulting in reduced resistance to flow and aids in the maintenance of resting blood flow.

4.3.2 Physiological Model

As previously described, Sundby demonstrated a sustained increase in local blood velocity five minutes after INP had ceased, in the absence of central haemodynamic effects (Sundby et al 2017). This suggests physiological changes and in particular, the release of vasoactive substances may be involved in the body's response to INP. One important component of the blood vessel wall – the endothelium, directly releases vasoactive substances and therefore may be involved in the sustained effects of INP. As highlighted previous, Holder has recently provided evidence to suggest endothelial activity may be altered upon exposure to INP in the upper limbs (Holder et al 2019).

Patients with PAD have reduced flow-mediated dilation (FMD), a direct measure of arterial endothelial function, compared to age-matched healthy controls (Brevetti et al 2003). Whether INP can improve endothelial function in PAD patients remains unknown. Although current vasodilator drugs are utilised by clinicians, with varying success, non-pharamalogical stimulation of the endothelium and enhancement of vasodilation would be hugely beneficial in the treatment of atherosclerotic PAD. In these patients, the endothelium is not stimulated as often due to obstructed flow leading to reduced vasodilator activity and damaged endothelium. It can be postulated that the repetitive application of INP, which elicits arterial inflow, stimulates the endothelium to release vasoactive substances (NO) causing vasodilation. The endothelium also plays an essential role in angiogenesis, the formation of new blood vessels from pre-existing vessels allowing for collateral blood circulation to ischaemic tissue. This process is triggered by hypoxia, present in patients with ischaemic arterial disease due to stenosis and is mediated by nitric oxide. Endothelial activation resulting in vasodilation and angiogenesis together would be hugely beneficial in amelioration of arterial disease symptoms by increasing blood flow in the area through both vasodilator enhanced flow and collateral flow. Furthermore, recently evidence has suggested that flowmotion is important in supplying oxygen and nutrients to tissues (Tsai et al 1933, Aalkjaer et al 2011). Applying INP creates oscillations in blood flow, as observed by Sundby, mimicking the naturally occurring flowmotion. These artificial oscillations in flow may elicit shear-stress endothelial activation leading to release of NO and other vasoactive substances.

Taken together, this ‘physiological model’ theorises that during INP application, there is increased shear stress exerted on the endothelium due to a local increase in blood flow triggering endothelial activation. Indeed, Greenfield demonstrated on two separate occasions that applying pressure to the lower and upper body brought about a local increase in blood flow due to vasodilation of the blood vessels through endothelial activation (Greenfield & Patterson 1954, Coles et al 1956). In a cell culture model, pulsatile shear stress evoked a large increase in the production of prostacyclin, a vasodilator released by the endothelium, compared to unexposed control cells (Frangos et al 1985). The same model has been used to establish increased NO production in response to shear stress (Cooke et al 1990).

It is clear the physiological model has strong evidence behind it, heavily involving the endothelium, however the theory remains unproven. The physiological model may provide

explanation for the sustained effects of INP on blood flow observed by Sundby et al through vasodilator release upon endothelial activation. Further experimentation is required to explore how INP therapy may alter both macro and microcirculatory endothelial function in healthy and diseased physiology.

Chapter 5: Methodology

5.1 Regulatory approvals

This study was approved and conducted in accordance with The Declaration of Helsinki. Before recruitment could commence, approval of all study documents including participant information sheets (PIS), protocol, informed consent forms, invitation letter, pain chart and GP letters was required. Approvals were obtained from Sponsors (University of Dundee and NHS Tayside), NHS Tayside Research & Development (R&D) and the Research Ethics Committee (REC) (REC Reference number: 19/ES/0005). All approvals were in place on 31st January 2019. For the cold hands study, ethical approval was gained from the University of Dundee School of Medicine Research Ethics Committee on the 15th November 2019 (SMED REC Number 19/131).

All participants were given a relevant information sheet which included extensive information relating to the aims of the study, study procedures, withdrawal procedures and data management (Appendix 1, 2, 3 & 4). Participants had no obligation to take part, the decision was down to each individual. Participants were able to withdraw from the study at any time. If a patient chose to take part in the study, their normal medical care was not affected in any way. All personal data was anonymised, and each participant given a unique participant identification number.

Study measurements were recorded on a paper case report form (CRF) which did not hold either the participants name or initials. CRFs were stored in a locked office and data was transcribed onto a Microsoft Excel database and saved onto a secure network.

5.2 Study design

Three separate groups were assessed. Healthy volunteers were recruited. Patients with PAD, and in particular suffering from critical limb ischaemia, were recruited. Both healthy volunteers and cold hand volunteers were recruited to the cold hand study (Figure 5.1).

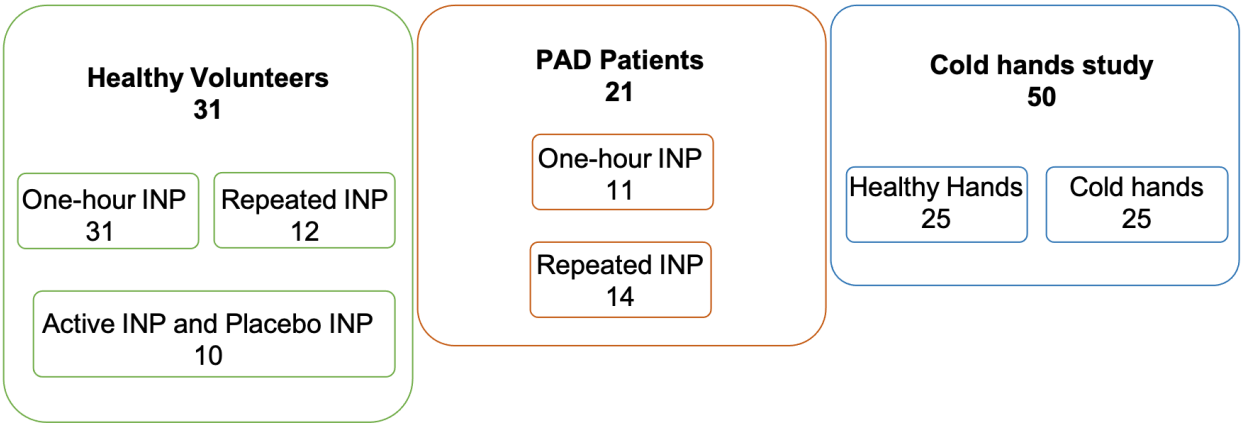

Figure 5.1. ***Study groups.*** Three study groups were assessed. 31 healthy volunteers were recruited. 31 underwent one-hour INP and 12 underwent repeated INP application. 10 healthy volunteers were assessed with both the active and placebo INP device. 21 PAD patients were recruited. 11 underwent one-hour INP and 14 received repeated INP application. 50 volunteers were recruited to the col hand study: 25 healthy volunteers and 25 cold hand volunteers.

5.2.1 Sample size calculation

Based on previous work exploring mechanisms of changes in vascular function using various interventions, it was evaluated that n=25 would provide at least 80% power and 5% significance to detect a 20% before and after difference within subjects in the vascular function markers included in this study, endothelial function and arterial stiffness. Previous studies evaluating the effects of an intervention on vascular function, namely endothelial function and vascular stiffness, have detected significant changes with a sample size of n=15 (Bagabir, 2019). Meanwhile studies conducted with Flow-Ox in PAD patients investigating clinical markers including walking distance have utilised a sample size of n=34 in active and sham groups (Hoel et al 2021a).

5.2.2 Recruitment

Recruitment for all groups took place between February 2019 and June 2021. There was a six-month halt to recruitment between March 2020 and September 2020 due to the COVID-19 pandemic.

Healthy volunteers were recruited mainly from the staff and student population within the School of Medicine according to the inclusion and exclusion criteria using poster

advertisements and e-mail circulations. Interested individuals were able to read a participant information sheet (appendix 2) and ask any questions before consenting to take part in the study. Inclusion criteria for healthy volunteers were: (1) no current or previous diagnosed cardiovascular illness, (2) able to provide written informed consent (3) >18 years of age. Exclusion criteria were (1) pregnant women. Pregnancy testing was performed on pre-menopausal women to confirm non-pregnancy prior to participation.

Eligible PAD patients were identified by members of the Vascular Team, Ward 12, Ninewells Hospital, Dundee, under the supervision of the Consultant Vascular Surgeons according to the inclusion and exclusion criteria and followed up by the Principal Investigator. Patients were given a patient information sheet, invitation letter and reply slip (Appendix 1 and 5 respectively). Patients were given a minimum of 24 hours to consider their participation prior to informed consent being obtained. Interested patients completed and returned the reply slip to the principal investigator. Inclusion criteria for PAD patients were: (1) PAD diagnosis, (2) were not due to undergo any major surgery or treatment within the next 4-8 weeks, (3) able to give written informed consent, (4) were mobile enough to be able to use the device themselves in their own homes, (5) >18 years of age. Exclusion criteria were (1) unable to give written informed consent and (2) deep vein thrombosis.

For the cold hands study, all volunteers were recruited from the staff and student population within the School of Medicine using poster advertisements and e-mail circulations. Eligibility was assessed according to the inclusion and exclusion criteria for each group. Inclusion criteria for healthy volunteers were: (1) >18 years of age, (2) able to give written informed consent, (3) no current or previous diagnosed cardiovascular disease (including coronary heart disease, congenital heart conditions and hypertension). Inclusion criteria for cold hands group were: (1) >18 years of age, (2) able to give written informed consent, (3) described symptoms of cold hypersensitivity including changes in colour of the digits and/or tingling or pain upon cold exposure. Pregnant women were excluded from taking part in both groups.

5.2.3 Study sample

31 healthy volunteers were recruited, mostly from the student population within the School of Medicine, University of Dundee.

In total 52 eligible PAD patients were approached to take part in the study. Of these, 24 consented to taking part in the study. Reasons for patients deciding not to take part included difficulty in travelling to and from the hospital for study visits, an inability to use the device independently for the required time and being schedules for intervention within the following 4 weeks. Recruitment of non-option critical limb patients proved to be more difficult than initially anticipated due to several reasons. The majority of these patients tend to undergo emergency intervention upon first presentation to the clinical team and were therefore unable to use the device for a minimum of 4 weeks. Out of the 24 patients who were consented, 3 patients had to be excluded from data analysis due to failure to return for repeat assessments. 15 out of 21 patients completed between 4-8 weeks of INP application at home. Due to the COVID-19 pandemic, it was not possible for a number of patients to return for repeat assessments after 4-8 weeks. It was possible for pain scores to be collected from these patients over the phone. Data collected from these patients at baseline and after one INP session was included in the data analysis.

50 participants were recruited to the cold hands study: 25 healthy volunteers and 25 cold hand participants. These participants were recruited mainly from the student and staff population of the School of Medicine. All cold hand participants exhibited Raynaud's-like symptoms including cold hypersensitivity, a change in finger colour and/or pain upon exposure to cold. The two groups were sex matched.

5.3 Study procedures

All participants were instructed to not consume any caffeine for at least 4 hours prior to the study visit as caffeine is known to elicit increases in blood pressure and blood flow in some vessels throughout the body.

Healthy volunteers were assessed at three time points (Figure 5.2).

- Baseline
- One INP session
- After repeated INP application for 5 days

Patients were assessed at three time points (Figure 5.3).

- Baseline
- One INP session
- After repeated INP application for up to 8 weeks

Volunteers in the cold hands study were assessed at two time points (Figure 5.4).

- Baseline
- One INP session

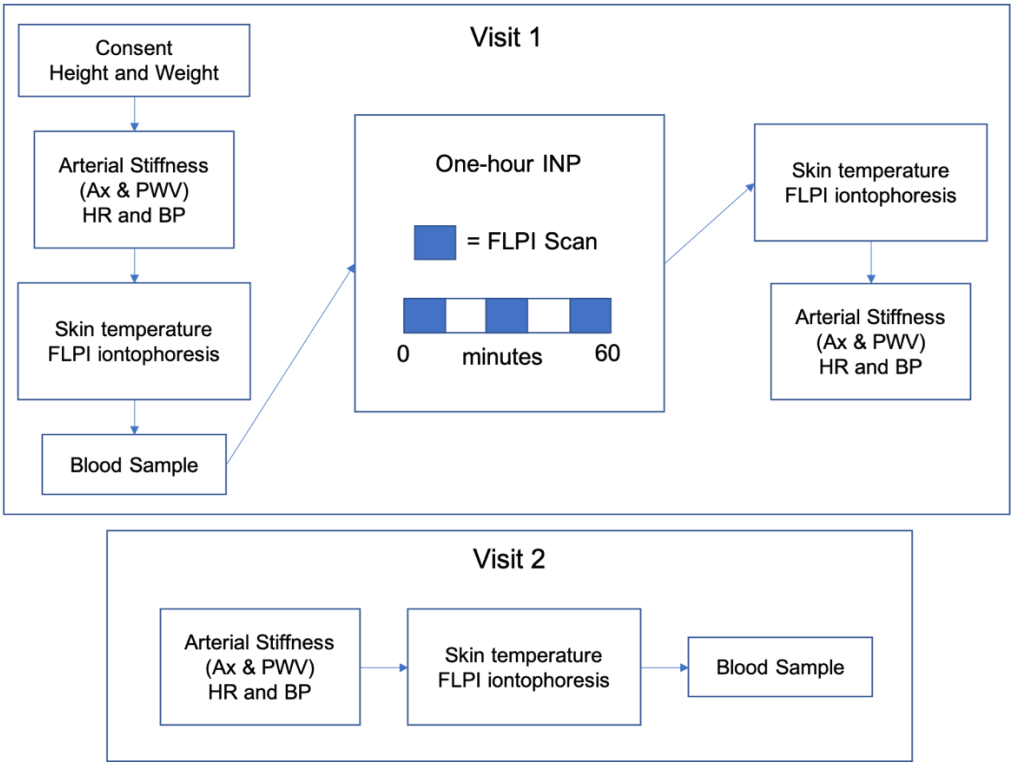

Figure 5.2. ***Study procedures timeline for healthy volunteers.*** Healthy volunteers were assessed during two separate visits. Visit one started with consent being obtained followed by measurement of height, weight, arterial stiffness, skin temperature, full field laser speckle contrast imaging (FLPI) and iontophoresis followed by collection of a blood sample. Volunteers then used the Flow-Ox device to deliver INP to the foot for one-hour and FLPI scans were carried out at three time points during INP application: 0-10 minutes, 25-35minutes and 50-60 minutes. After INP application, skin temperature, FLPI and iontophoresis, arterial stiffness and central haemodynamics were assessed again. Visit two consisted of repeated assessments as in visit 1. AIx = augmentation index, PWV = pulse wave velocity, HR = heart rate, BP = blood pressure.

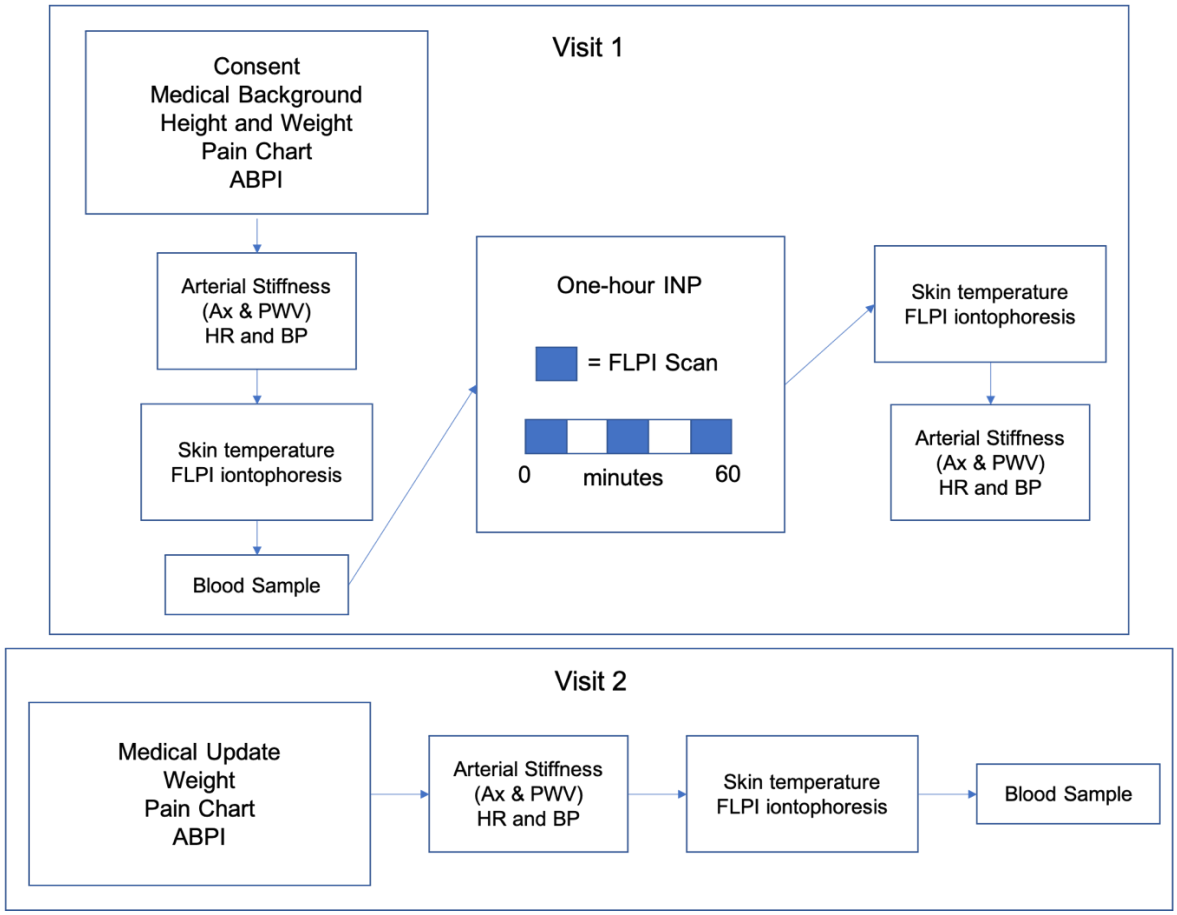

Figure 5.3. ***Study procedures timeline for PAD patients.*** PAD patients were assessed during two separate visits. Visit one started with consent being obtained followed by review of the patient's medical history and measurement of height, weight, pain chart, ABPI, arterial stiffness, skin temperature, full field laser speckle contrast imaging (FLPI) and iontophoresis followed by collection of a blood sample. Patients then used the Flow-Ox device to deliver INP to the foot for one-hour and FLPI scans were carried out at three time points during INP application: 0-10 minutes, 25-35minutes and 50-60 minutes. After INP application, skin temperature, FLPI and iontophoresis, arterial stiffness and central haemodynamics were assessed again. Visit two consisted of repeated assessments as in visit 1. AIx = augmentation index, PWV = pulse wave velocity, HR = heart rate, BP = blood pressure.

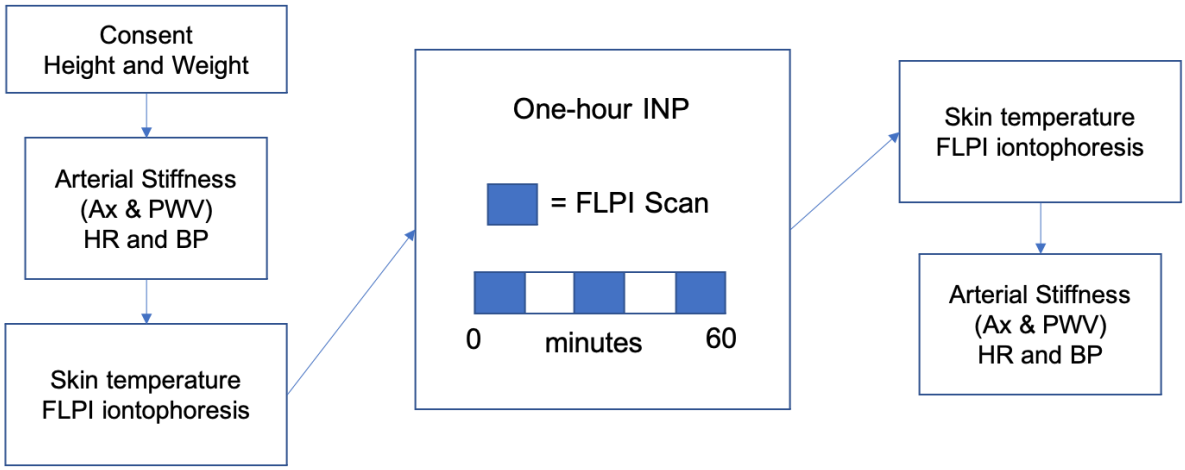

Figure 5.4. ***Study procedures timeline for cold hands study.*** Volunteers in the cold hands study were assessed during one visit. First, consent was obtained followed by measurement of height, weight, arterial stiffness, skin temperature and full field laser speckle contrast imaging (FLPI) and iontophoresis. Participants then used the Flow-Ox device to deliver INP to the hand for one-hour and FLPI scans were carried out at three time points during INP application: 0-10 minutes, 25-35minutes and 50-60 minutes. After INP application, skin temperature, FLPI and iontophoresis, arterial stiffness and central haemodynamics were assessed again. AIx = augmentation index, PWV = pulse wave velocity, HR = heart rate, BP = blood pressure.

5.3.1 Informed Consent

Informed consent was obtained at the first study visit to the research department by a Good Clinical Practise (GCP) qualified researcher prior to any study procedures taking place.

5.3.2 Room set up

All measurements for all participants were conducted in the same room with a set temperature of 22°C. Participants were allowed approximately 10 minutes to acclimatise once entering the room before study assessments were conducted. Participants were either seated or supine for study assessments.

5.3.3 Assessment of vascular function

Vascular function of participants was assessed through several measurements. Blood perfusion was measured using full field laser speckle contrast imaging (FLPI) and, alongside

iontophoresis, this method was used to assess endothelial function. Arterial stiffness was assessed by measuring augmentation index (AIx) and pulse wave velocity (PWV).

5.3.3.1 Central haemodynamics

Central haemodynamics assessed included heart rate, systolic blood pressure and diastolic blood pressure. These measurements were taken using the SphygmoCor (AtCor Medical, UK) as described in section 5.3.3.4.

5.3.3.2 Blood perfusion: Full Field Laser Speckle Contrast Imaging (FLPI)

FLPI (FLPI2, Moor Instruments, UK) was used to assess microvascular blood perfusion. The FLPI system measures blood perfusion of the skin microvasculature by utilising laser speckle contrast imaging (LSCI) techniques. LSCI can non-invasively produce a perfusion map of large areas of skin in real-time. LSCI is based on the principle that laser light projected onto the skin produces backscattered light and produces a random interference patter which is detected. This is called the speckle pattern. Fluctuations in this speckle pattern occur when blood cells move within the blood vessels thus providing an output value equivalent to perfusion. A colour-coded real-time image or video (25 frames per second) is obtained, and flux measured (Figure 5.5). As perfusion is not measured quantitively, it was expressed as blood flux in arbitrary perfusion units (au).

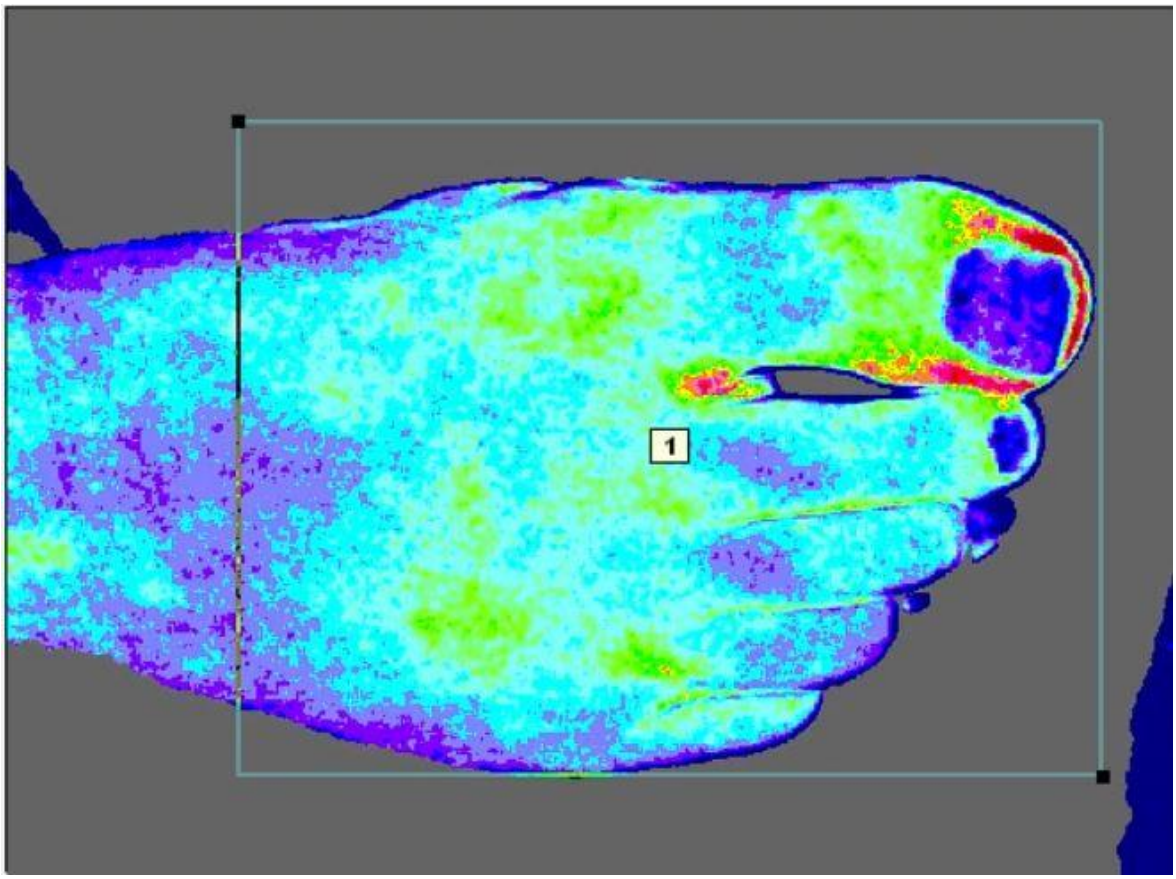

Figure 5.5. ***Example image obtained from Full Field Laser Speckle Contrast Imaging (FLPI).*** An example image of the dorsum of the foot obtained from FLPI. The output gives a colour-coded image with red depicting areas of very high blood perfusion and dark blue depicting areas of low blood perfusion.

FLPI is a non-invasive method to measure blood perfusion however measurements obtained by FLPI can be affected by a number of things including biological zero, time of day, spatial heterogeneity and movement artefacts. Biological zero refers to the detectible signal which is still present even when blood flow is totally occluded. Some researchers suggest biological zero value should be subtracted from perfusion values obtained however this has not been shown to alter overall results. Time of day which the measurements are obtained can affect results as blood perfusion fluctuates within the body throughout the day. In this study, participants were assessed, where possible, at the same time of day if being assessed on separate days. Blood vessels are highly heterogenous between different sites on the body and therefore care was taken to ensure repeat measurements were conducted on the exact same area of skin as baseline. When a participant moves, the FLPI imager picks up on this and a large spike in perfusion is recorded creating movement artifacts. In order to reduce this, participants seated or lying comfortably before assessment began and were asked to remain

as still as possible and not to speak during scanning. When FLPI scans were being performed on the foot, the participant's foot was rested on a stool and cushion to stabilise the foot (Figure 5.6, Figure 5.7).

Figure 5.6 ***Full field laser speckle contrast imaging (FLPI) set up with Flow-Ox for assessment of blood perfusion in the foot.*** In order to assess blood perfusion of the dorsum of the foot while INP was applied, the participant's foot was placed inside the pressure chamber and rested on a footrest. The pressure chamber was rested on a small stool. The FLPI imager was located above the surface of the foot and skin blood perfusion measured.

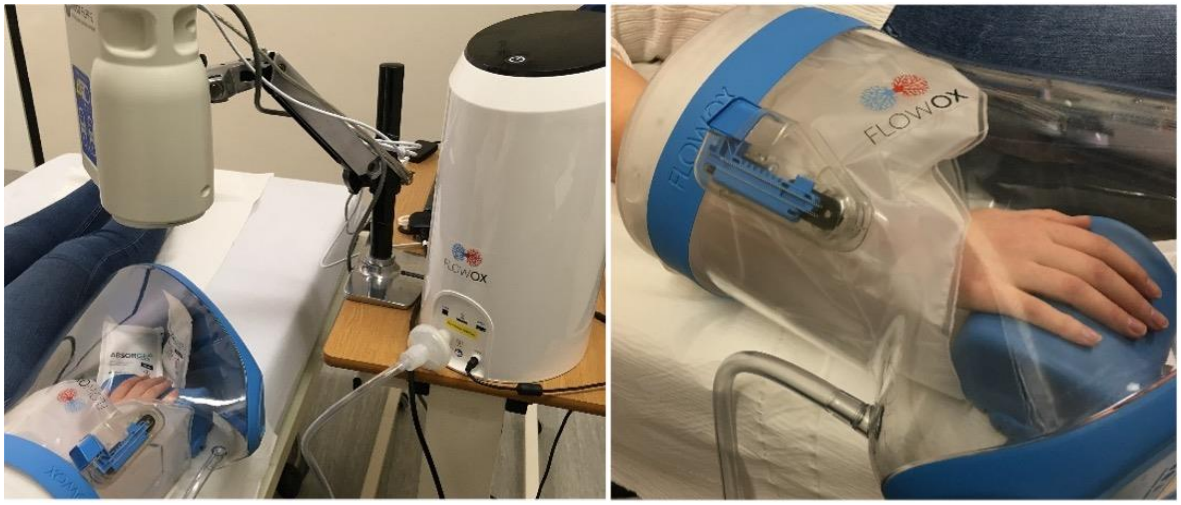

Figure 5.7 ***Full field laser speckle contrast imaging (FLPI) set up with Flow-Ox for assessment of blood perfusion in the hand.*** To assess blood perfusion of the dorsum of the hand and fingers while INP was applied, the participant's hand was placed inside the pressure chamber and rested on a hand rest. The FLPI imager was located at an appropriate distance from the surface of the hand and skin blood perfusion measured.

5.3.3.3 Endothelial function: Iontophoresis

Iontophoresis is a technique commonly used to investigate endothelial function in both humans and animals. The method involves using an electric current to drive ions through the skin, into the microcirculation. Iontophoresis has been described in greater detail in section 2.2.3. Endothelial function was assessed to evaluate the effects of INP on microvascular function at different time points relative to INP delivery.

Delivery of ions acetylcholine (ACh) and sodium nitroprusside (SNP) by iontophoresis can be affected by numerous factors including skin resistance and the Galvanic effect. Skin resistance, which can differ greatly between individuals and location of the body, can influence ion delivery. Ramsay found high skin resistance results in a reduced response to both ACh and SNP (Ramsay 2002). In order to reduce the effects of skin resistance on the measurement, the skin was epidermal stripped and cleaned to remove dead skin cells and oils using adhesive tape and an alcohol wipe. The Galvanic effect refers to the induction of cutaneous vasodilation by the vehicle in which the vasoactive chemicals are diluted and not induced by the vasoactive chemicals themselves. Vehicles which induce dermal vasodilation include sodium chloride and tap water. Some researchers subtract the vehicle response from flux values however this has been shown to rarely effect study results.

1% ACh and SNP solution were prepared by measuring 0.1g of ACh or SNP (Sigma Aldrich) out using an analytical scale and dissolved in 10ml of deionised water in a 10ml conical tube. As SNP is light sensitive, the conical tube was covered with foil at all times. Delivery of ions ACh and SNP can be affected by numerous factors including skin resistance and the Galvanic effect. Skin resistance, which can differ greatly between individuals and location of the body, can influence ion delivery. Ramsay found high skin resistance results in a reduced response to both ACh and SNP (Ramsay 2002). In order to reduce the effects of skin resistance on the measurement, the skin was epidermal stripped and cleaned to remove dead skin cells and oils using adhesive tape and an alcohol wipe.

The skin was prepared using adhesive tape and an alcohol wipe. Two small circular electrode chambers attached to the skin using adhesive tape (Figure 5.8). The circular electrode chambers had an internal diameter of 20mm. The chambers were washed and sanitised between each measurement and each participant to avoid cross contamination. 2ml of ACh and SNP were added to each chamber and sealed with the chamber lid. Since ACh is positively charged and SNP negatively charged, the ACh electrode chamber was connected to the positive lead and SNP to the negative. The leads connected to the iontophoresis controller (MIC2, Moor Instruments, UK). On the FLPI Imager software (Moor instruments, UK), areas of interest were selected, and the camera was focussed before beginning the scan. A baseline measurement was obtained for 2 minutes before the iontophoresis controller was turned on and delivered a current of 100uA for 5 minutes after which the iontophoresis controller was switched off and post-current measurement obtained until perfusion had plateaued.

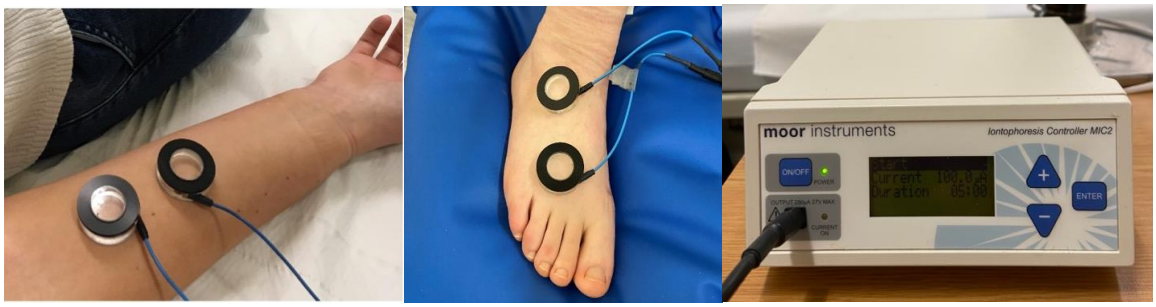

Figure 5.8. ***Iontophoresis electrode chamber placement on the forearm and foot.*** Electrode chambers were attached to the skin using adhesive tape. The electrodes were connected to the iontophoresis controlled (Moor Instruments, UK) which controlled ion delivery.

5.3.3.3.1 Analysis of FLPI measurements

Analysis of the data collected with the FLPI was completed with the FLPI2-Review V5.0 software (Moor instruments, UK). Areas of interest were selected on the FLPI trace included baseline and peak perfusion. Peak perfusion was defined as the highest point of each trace. Median values for these areas of interest were recorded. Percentage change in perfusion from baseline to peak was calculated as:

$$\frac{(\text{Peak Value} - \text{Baseline Value})}{\text{Baseline Value}} \text{ X } 100$$

5.3.3.4 Arterial stiffness

The background and theory of arterial stiffness is described in detail in section 2.4.
Arterial stiffness was assessed to evaluate the effects of INP on systemic macrovascular function, away from the site of INP application. Arterial stiffness was measured by assessing augmentation index (AIx) and pulse wave velocity (PWV).

5.3.3.4.1 Pulse wave analysis: Augmentation index (AIx)

Pulse wave analysis was carried out by measuring augmentation index (AIx). SphygmoCor (AtCor Medical, UK) was used to measure AIx in participants. The software required input of participant ID, date of birth, gender and height before measurements could be carried out. An inflatable cuff was placed around the upper arm of participants ensuring correct positioning relative to the brachial artery in order to capture the brachial waveform (Figure

5.9). A top of thin material was allowed to be worn by participants as this did not affect measurement. The SphygmoCor software automatically inflated the cuff and recorded brachial waveform as well as patient heart rate and blood pressure. AIx is automatically calculated by the software as the difference in systolic peaks of the incident and reflected wave divided by pulse pressure multiplied by 100. The software displayed the accuracy of the measurement in the form of an operator index. If the operator index was not sufficient, the test was repeated.

Figure 5.9 ***SphygmoCor Assessment of brachial augmentation index.*** SphygmoCor (AtCor Medical, UK) was used to assess arterial stiffness in participants. For assessment of brachial augmentation index (AIx), the pressure cuff was placed around the upper arm of the participant and tubing placed in line with the approximate location of the brachial artery. The machine automatically inflates the pressure cuff and AIx recorded.

5.3.3.4.2 Pulse wave velocity (PWV)

SphygmoCor (AtCor Medical, UK) was used to measure pulse wave velocity (PWV). An inflatable cuff was placed around the upper thigh ensuring correct positioning relative to the femoral artery in order to capture the femoral waveform. Several distance measurements were taken in order to calculate the pulse wave transit distance; carotid artery to sternal notch, sternal notch to top of thigh cuff and femoral artery to top of cuff. The carotid pulse was located in the participant's neck and a tonometer placed over the carotid artery to capture the

carotid waveform. The SphygmoCor software automatically inflated the femoral cuff when a carotid waveform had been located and deflated after both waveforms had been recorded accurately. The software automatically calculated PWV as distance divided by pulse transit time and expressed the value in meters per second (m/s) (Figure 5.10). Operator index was recorded and if too low, the machine required a repeated measurement to calculate PWV.

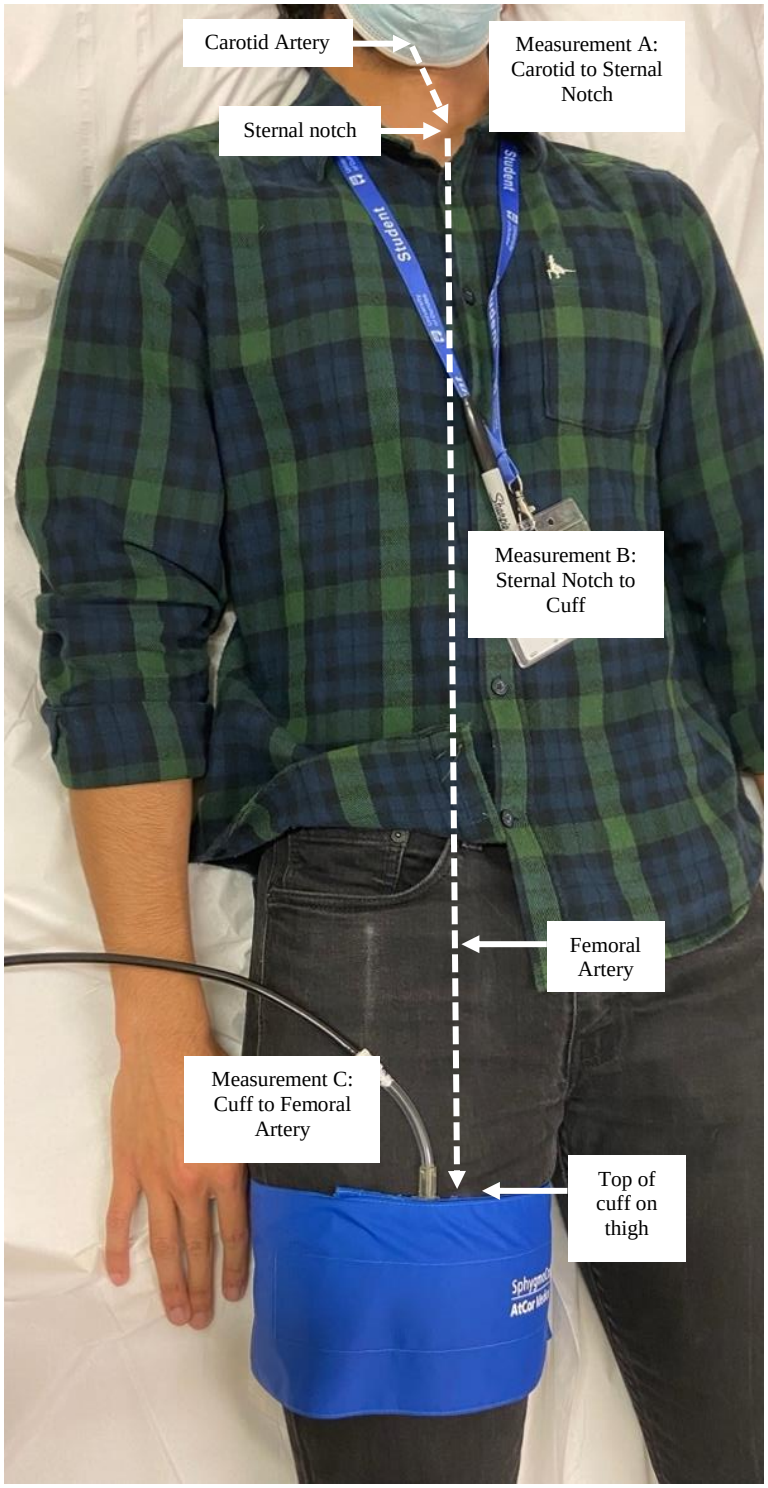

Figure 5.10 ***SphygmoCor assessment of pulse wave velocity.*** SphygmoCor (AtCor Medical, UK) was used to assess pulse wave velocity in participants. For assessment of carotid-femoral pulse wave velocity, the pressure cuff was placed around the participant's thigh followed by a number of measurements: (A) carotid artery to sternal notch, (B) sternal notch to top of the cuff and (C) top of the cuff to femoral artery. These measurements were inputted to the software and pulse wave velocity calculated automatically by the machine.

5.3.4 Ankle brachial pressure index (ABPI)

Ankle brachial pressure index (ABPI) is the ratio of blood pressure at two points typically the brachial artery in the arm and dorsalis pedis or posterior tibial of the foot. ABPI is routinely used in the diagnosis of PAD as described in section 3.1.3.

In healthy individuals, systolic blood pressure increases in the periphery. For example, blood pressure in the ankle is slightly greater than in the arm. When a haemodynamically significant stenosis is present, systolic blood pressure distal to the stenosis falls and therefore peripheral blood pressure is less than central pressure. An ABPI between 0.9 and 1.4 is considered healthy while an ABPI <0.9 is indicative of PAD. ABPI for CLI patients are usually extremely low (<0.4) and reflects the obstructed blood flow.

ABPI was assessed in PAD patients by measuring systolic blood pressure in the brachial artery of both arms and dorsalis pedis or posterior tibial of the foot using a handheld 8MHz ultrasound blood velocity detector and blood pressure cuff. ABPI was calculated as foot diastolic pressure divided by the highest brachial value.

5.3.5 Pain chart

A pain chart was used to assess PAD patient's pain levels. Patients were asked to rate their pain from 0 to 10 using a visual analogue scale (VAS) for pain measurement. The scale ranged from 0 to 10 with 0 being no pain at all to 10 being the worst pain they have ever felt (Appendix 6). When reporting their second pain score after repeated INP application, patients were unable to see the pain score they reported at baseline so as not to influence their decision.

5.3.6 Application of intermittent negative pressure (INP): Flow-Ox

INP was applied to participants using the Flow-Ox system, a CE marked non-invasive medical device designed, developed, and provided by Otivio AS, Norway (Figure 5.11). The Flow-Ox device is specially designed to treat patients with PAD and has been previously

extensively tested in this patient group yielding positive clinical outcomes as previously described (Chapter 4 Section 2).

Figure 5.11. ***Flow-Ox device.*** The Flow-Ox device (Otivio AS, Norway) was used to delivery intermittent negative pressure to study participant's foot or arm. The device is comprised of a pressure chamber (A) and control unit (B). The pressure chamber is composed of plastic to withstand changes in pressure and contains a replaceable rubber seal and padding to ensure and air-tight environment and limit pressure points. The hose connects the pressure chamber to the control unit, which delivers the negative pressure cycles.

The Flow-Ox device comfortably accommodates the foot and calf. The Flow-Ox is made up of two separate components – the control unit and pressure chamber. The control unit and pressure chamber are portable and therefore allow patients to take the device home and use it independently with ease. The control unit is responsible for regulation of negative pressure within the pressure chamber and delivery of negative pressure. The negative pressure cycle of 10 seconds negative pressure and 7 seconds no pressure is pre-programmed in the control unit. The control unit also has a display screen which displays time of usage and any error

messages. If air leakage was present, the control unit displays an error message to alert the user to fix the problem. The control unit also recorded when the device was being used. This data can be downloaded by the researcher and viewed on Flow-Ox software on a computer. The pressure chamber is composed of polyethylene plastic and can withstand changes in pressure without deforming. The seal is elastic to allow for it to be stretched to accommodate and mould to the leg. The seal ensures the environment within the pressure chamber is separate to the environment out with thus maintaining the required negative pressure environment. The seal is approved for prolonged contact with the skin. The padding is designed to stabilise the calf while negative pressure is delivered and to limit pressure points. The hose which connects the control unit to the pressure chamber is fitted with a HEPA air purifier filter to control contamination and prevent infection. Each pressure chamber was used only once to prevent cross-contamination and therefore each participant received a brand-new pressure chamber. As control units did not come into contact with the participant's foot, control units were decontaminated between participants and used multiple times.

The change in venous pressure experienced during delivery of -40mmHg is thought to be similar to the change experienced when an individual moves from a supine to standing position. The use of this device in patients and healthy individuals is deemed safe with no major side effects or adverse events being reported in any of the studies utilising the device. Use of the device in pregnant women is currently untested and therefore this group was excluded from this study.

Participants were fully trained to use the Flow-Ox system so they could use it themselves in their home. Patients were asked to use the device daily for between 4 and 8 weeks and healthy volunteers were asked to use the device daily for 5 days. Previous studies using Flow-Ox have tested the optimal daily treatment length to see clinical benefits with results indicating 2 hours per day (Sundby et al 2016). When patients were ready to use the device, they were asked to be comfortably seated and to place the experimental leg into the pressure chamber ensuring the rubber seal was placed around the calf to prevent air leakage from the pressure chamber. Once turned on, the control unit automatically controlled pressure within the pressure chamber in a cyclical protocol of 10 seconds -40mmHg followed by 7 seconds no pressure. A digital timer appeared on the control unit which counted upwards, so patients knew how long they had been using the device. The timer automatically reset overnight so

each day started at 00:00. Participants were asked to use the system for two hours per day which could be in one sitting or split into two one-hour session. Participants decided what time of day they decided to use the device as long as the total time each day was two hours. Patients were allowed to wear a sock or dressings inside the boot while it was in use, this did not interfere with INP application. If the patient experienced any problems with the device while using it at home, they were able to contact the principal investigator via telephone and assistance would be given.

For the cold hands study, the same Flow-Ox device was used however a small number of alterations were made to the protocol for use on the arm. The pressure chamber was positioned in a way that it could accommodate the hand and lower arm comfortably while supine (see Figure 5.5). The position of the pressure chamber did not affect use of the device. The participant's hand was rested on a hand rest.

A placebo, or sham, Flow-Ox device was used for part of this study. This placebo device is produced and appears exactly the same as the active -40mmHg device however a negative pressure of -10mmHg is delivered. A previous study has demonstrated that delivery of -10mmHg is not sufficient to induce any physiologically significant changes in blood perfusion and is therefore appropriate to be used in a placebo device (Hoel et al 2019).

5.3.6.1 Compliance

The control unit of Flow-Ox automatically recorded when the device was being used. Prior to the patient taking the device home, the serial number of the device given was recorded. Upon return of the device, the usage data was downloaded onto a USB stick and uploaded to a secure computer and viewed using the Flow-Ox software (Otivio AS, Norway). The software allowed the investigator to view which days the device had been used and for how long. Specific date ranges could be selected and viewed. The actual total number of days used, and total number of hours used were counted and divided by expected total number of days and time used. Compliance was calculated as the percentage of actual usage.

5.3.7 Skin temperature

Skin temperature was measured using a handheld mini-IR thermometer (RS, 438-3365). Skin temperature was measured to help determine if any changes in perfusion which were recorded could be attributed to a change in local skin temperature. Furthermore, superficial microvessels are affected by ambient temperature therefore it was essential to ensure skin temperature was adequate before assessment.

5.3.8 Blood sampling

To allow for later blood biomarker analysis, approximately 40ml of venous blood was obtained from participants during the first visit and last visit. BP Vacutainer® Safety-Lok™ blood collection kit was used to collect blood into one 9ml Purple Cap Vacuette® K3EDTA blood collection tube and one 9ml Red Cap Vacuette® Z Serum Sep Clot Activator Tube. The Purple Cap tube was used to extract plasma, and one Red Cap tube was used to extract serum.

Biomarkers analysed were intercellular adhesion molecule-1 (ICAM-1), matrix metalloproteinase-8 (MMP-8), vascular cell adhesion molecule (VCAM-1), interleukin 1 alpha (IL-1a), endothelial selectin (E-selectin) and human myeloperoxidase (MPO). These biomarkers were chosen as previous studies have demonstrated elevated levels in PAD or a link between levels of the biomarker and disease and therefore would provide a good indication of change in disease status if levels were altered by INP.

5.3.8.1 Plasma and Serum Extraction and storage

The purple and red cap tubes were spun in a centrifuge at 3500rpm for 10 minutes to separate serum and plasma from red blood cells. Serum and plasma were aliquoted into 1ml samples and stored at -80°C.

5.3.8.2 Quantikine ELISA for Human Myeloperoxidase (MPO)

Quantikine ELISA for Human Myeloperoxidase (MPO) procedure was performed as detailed in the manufacturer's instructions (R&D Systems, USA).

Serum collected from participants was used to measure MPO levels. Samples which had been stored at -80C were thawed and allowed to reach room temperature. All reagents were allowed to reach room temperature. Human MPO standard was reconstituted with deionized water to produce a stock solution of 100ng/ml. This was allowed to sit for 15 minutes to allow proper dissolving.

Serum samples were prepared in 1.2ml microtubes. Samples were diluted 50-fold. 900ul Diluent RD6-58 was mixed with 10ul of each serum sample and briefly vortexed. 50ul of this diluted sample was mixed with 200ul Diluent RD6-58 and vortexed briefly.

Human MPO standard, which had previously been reconstituted, was used to yield a standard curve through serial dilution. 8 standards were made up as follows:

- 1: 900ul Diluent RD6-58 + 100ul standard
- 2-7: 500ul Diluent RD6-58 + 500ul from previous standard.
- 8: 500ul Diluent RD6-58

100ul Assay Diluent RD1-27 was added to each well followed by 50ul of either standard, control or sample in duplicate. An adhesive strip was placed over the top of the plate to seal it. The plate was left to incubate the room temperature on a horizontal orbital shaker set to 550rrmp for 2 hours. 20ml of wash buffer concentrate was mixed with 480ml deionized water to make up the wash buffer. After incubation, the wells were washed four times with pre-prepared wash buffer. 200ul Human MPO conjugate was added to each well and recovered with an adhesive strip. The plate was left to incubate again at room temperature for 2 hours, with shaking as before. After incubation, the wells were washed as before. 200ul of substrate solution was added to each well and the plate left to incubate for 30 minutes at room temperature on the bench, covered to protect from the light. After incubation, 50ul of stop solution was added to each well and mixed until a colour change was evident. The optical density of each well was obtained within 30 minutes of stop solution addition using a microplate reader set to 450nm. For each duplicate standard, control and serum sample, the average was calculated and subtracted from the blank standard reading.

A standard curve was produced using computer software (SkanIt Software Version 2.4.3.37, Thermo Fisher Scientific). The serum samples were compared against the standard curve to determine the MPO concentration of each sample. The concentration of MPO was multiplied by the dilution factor to give the actual concentration.

5.3.8.3 Magnetic Luminex Assay

Magnetic Luminex assay procedure was performed as detailed in the manufacturer's instructions (R&D Systems, USA). The kit was used to detect ICAM-1, IL-1alpha, MMP8, VCAM-1 and E-selectin.

Plasma samples collected from participants which had been stored at -80C were thawed and allowed to each room temperature then centrifuged at 16000xg for 4 minutes. Plasma samples were diluted in a 4-fold dilution. 30ul of sample was added to 90ul Calibrator Diluent RD6-40 and mixed thoroughly.

Standard cocktails A and B were reconstituted with Calibrated RD6-40 and allowed to sit for 15 minutes to allow for proper dissolving. Reconstituted standard cocktails were used to prepare eight standards to yield a standard curve through serial dilution as follows:

- 1: 800ul Calibrator Diluent RD6-40 + 100ul standard cocktail A + 100ul standard cocktail B.
- 2-7: 500ul Calibrator Diluent RD6-40 + 100ul from the previous standard.
- 8: 500ul Calibrator Diluent RD6-40

50ul of each standard or sample was added to each well of a 96-well R&D specialised plate. The microparticle cocktail was resuspended by vortexing and diluting 500ul with 5ml RD2-1 diluent. 50ul of the diluted microparticle cocktail was added to each well of the 96-well plate. The plate was covered with a foil plate sealer and incubated for 2 hours at room temperature on a horizontal orbital shaker set at 800rpm.

20ml of wash buffer concentrate was added to 480ml deionized water to prepare 500ml of wash buffer. A magnetic microplate device was used to wash the wells three times. Each

time, the liquid was removed, 100ul wash buffer added, allowed to stand for 1 minute then the liquid was removed again.

Diluted biotin-antibody cocktail was prepared by centrifuging the biotin-antibody cocktail for 30 seconds at 1000xg followed by diluting 500ul biotin-antibody cocktail with 5ml RD2-1 diluent. 50ul diluted biotin-antibody cocktail was added to each well. The plate was re-covered with a new foil plate sealer and incubated at room temperature on an orbital shaker set at 800rpm for 60 minutes. After incubation, the wells were washed three times as described previously. Streptavidin-PE was prepared by centrifuging the Streptavidin-PE vial for 30 seconds at 1000xg followed by diluting 220ul streptavidin PE concentrate with 5.35ml wash buffer. 50ul diluted Streptavidin-PE was added to each well and covered with a foil plate sealer. The plate was incubated at room temperature on an orbital shaker set at 800rpm for 30 minutes. After incubation, the wells were washed as previously described. The microparticles were resuspended in 100ul wash buffer and incubated at room temperature for 2 minutes on the orbital shaker set at 800rpm.

The median florescence intensity for each well was assessed within 90 minutes using Bio-Rad Bio-Plex 200 system. A standard curve was produced using computer software (Bio-Plex Manger V6.1). Plasma samples were compared against the standard curve to determine analyte concentration of each sample. The concentrations were multiplied by the dilution factor to give actual concentration.

5.4 Wavelet Analysis

The Moor FLPI2-Review V5.0 software includes an in-built wavelet analysis feature. Using this feature, wavelet analysis was carried out on FLPI traces recorded of participant's cutaneous foot perfusion at the different time points. The FLPI signal was separated into each of the six components according to frequency; cardiac (0.6-2 Hz), respiratory (0.145-0.6 Hz), myogenic (0.052-0.145 Hz), neurogenic/sympathetic (0.021-0.052 Hz), NO-dependent endothelial activity (0.0095-0.021 Hz) and NO-independent endothelial activity (0.005-0.0095 Hz). These components have been described previously in deeper detail (Section 2.3). The Moor software output gave a percentage contribution of each vasomotion competent to the overall signal obtained from FLPI. This value was recorded and used in analysis.

5.5 Statistical analysis

All statistical analysis of data were performed using IBM® SPSS ® Statistics 27 software package and Microsoft ® Excel.

Normality was assessed using Shapiro-Wilk test as this normality test is more appropriate for smaller sample sizes than other tests such as Kolmogorov-Smirnov. Data was considered to be normally distributed if significance > 0.05. Where data was considered to be normally distributed, parametric tests were used. Where data was considered to be of non-normal distribution, non-parametric tests were used. Parametric statistical tests performed included paired-samples t-test (when comparing the same group, between two time points), independent samples t-test (when comparing two groups) and one way analysis of variance (ANOVA) with post-hoc Bonferroni analysis where appropriate (when comparing the same group, at multiple different time points). Non-parametric statistical tests performed included Wilcoxon signed rank test (when comparing the same group, between two time points), Mann-Whitney U test (when comparing two groups) and Friedman test followed by post-hoc Wilcoxon signed rank test with Bonferroni adjustment as appropriate (when comparing the same group, at multiple different time points). Appropriate post-hoc analysis was carried out where required. Correlation analysis was carried out with Pearson correlation coefficient for normally distributed data and Spearman's correlation coefficient for non-parametric data. Data was presented as mean with standard deviations (SD) except for skewed data which was presented as median with interquartile range (IQR).

Chapter 6: Results

6.1 Foot Study: Healthy Volunteers

The effects of INP applied to the foot of healthy volunteers on both local and systemic vascular function was tested in the first instance to assess any changes to healthy physiology which may occur with application of INP. The Flow-Ox device, used to administer INP, was used safely throughout the study and no side effects or adverse events were reported in healthy volunteers.

Volunteers recruited were all healthy and free from any current or previous cardiovascular disease. In total, 31 healthy volunteers were recruited. 12 of these volunteers were assessed at three-time points; baseline, after one INP session and after repeated INP application for 5 days (group 1). This allowed investigation into the effects of repeated application of INP on healthy physiology. All 31 volunteers were assessed at two time points: baseline and after one INP session (group 2). 10 volunteers from group 2 returned for assessment with a placebo INP device at two-time points; baseline and after one placebo INP session (group 3). Any medication which volunteers were currently on was noted. These medications included the oral contraceptive pill (6 volunteers), antibiotics (1 volunteer), and a salbutamol inhaler for asthma (1 volunteer). The median age of healthy volunteers was 25 years old with ages ranging from 18 to 51. 10 volunteers were male and 21 females. No current smokers (cigarette, vape or other) were recruited to the study. All volunteers refrained from caffeine and large meals for at least 4 hours before the study assessments.

6.1.1 Group 1: Baseline, one INP session and five days INP

A total of 12 healthy volunteers were assessed at three-time points; baseline, after one INP session and after 5 days daily INP sessions. Baseline characteristics for these volunteers can be found in table 6.1.1. The median age of participants was 27.50 years old with the youngest being 22 years old and eldest being 51 years old. This group consisted of 4 males and 8 females.

Table 6.1.1: ***Baseline characteristics of healthy volunteers.*** Baseline characteristics for volunteers who underwent assessment at three time points. Normally distributed data is presented as mean (SD) and non-normally distributed data is presented as median (IQR). SD = standard deviation, IQR = interquartile range, BMI = body mass index.

Total number (n)	12
Age years **Median (IQR)** **(Min – Max)**	27.5 (10) 22-51
Sex	
Male n	4
Female n	8
Height cm **Mean (SD)**	172.92 (10.68)
Weight kg **Mean (SD)**	79.08 (14.09)
BMI kg/m^2 **Mean (SD)**	26.41 (4.36)

6.1.1.1 Central haemodynamics

Central haemodynamics were assessed in healthy volunteers by measuring systolic blood pressure, diastolic blood pressure and heart rate at each of the three time points. No significant changes in central haemodynamics were recorded over the three time points (Table 6.1.2).

Table 6.1.2: ***Recorded central haemodynamics in healthy volunteers.*** Vital central haemodynamics measured were heart rate, systolic blood pressure and diastolic blood pressure. Measurements were taken at baseline, after one INP session and after repeated INP sessions. All variables were normally distributed therefore repeated-measures ANOVA was used to assess significance. n = 12. SD = standard deviation, bpm = beats per minute.

	Baseline	**After one INP session**	**After repeated INP sessions**	**p-value**
Heart rate (bpm) Mean (SD)	65.29 (10.02)	62.23 (9.54)	64.08 (11.52)	0.195
Systolic Blood Pressure (mmHg) Mean (SD)	120.13 (9.59)	118.06 (9.14)	117.25 (6.24)	0.947
Diastolic blood pressure (mmHg) Mean (SD)	71.32 (8.40)	68.42 (7.58)	69.50 (5.93)	0.594

6.1.1.2 Arterial stiffness

In healthy volunteers, AIx and PWV were recorded at baseline, after one INP session and after repeated INP application (Table 6.1.3). AIx significantly decreased after one INP session from a baseline median of 15.00 to 10.00 after one INP session (p=0.002). AIx increased again to a median of 14.00 after repeated INP sessions however this was not significant when compared to after one INP session or baseline. When AIx was adjusted for heart rate, the same pattern was observed with a decreased after one INP session, versus baseline, followed by an increase back to baseline levels after repeated INP sessions however these differences were not statistically significant. Pulse wave velocity, a more direct measure of arterial stiffness, decreased slightly after one INP session and increased slightly after repeated INP sessions, compared to baseline. Repeated measures ANOVA revealed a significant difference between measurements at the three time points (p = 0.04). Post-hoc analysis revealed no significant difference between PWV values at baseline and after one INP session (p = 1.00) or baseline and after repeated INP (p = 0.50). However, PWV after one INP session and after repeated INP sessions were significantly different (p = 0.048) (Figure 6.1.1).

Table 6.1.3: ***Arterial stiffness measurements in healthy volunteers.*** Arterial stiffness was assessed by measuring augmentation index (AIx), AIx adjusted for 75bpm and pulse wave velocity (PWV) at the three different time points; baseline, after one INP session and after repeated INP sessions. Repeated measures ANOVA was used for normally distributed data with post-hoc analysis as required (a). Friedman test was used for non-normally distributed data alongside post-hoc Wilcoxon signed rank test with Bonferroni adjustment (b). n = 12. SD = standard deviation, IQR = interquartile range, AIx = augmentation index, m/s = metres per second.

	Baseline	**After one INP session**	**After repeated INP sessions**	**p-value**
Augmentation index (%AIx) Median (IQR)	15.00 (11.25)	10.00 (6.50)	14.00 (7.75)	0.014 [b]
Augmentation index@75bpm (%AIx75) Median (IQR)	8.00 (12.75)	1.50 (7.00)	9.50 (17.00)	0.088 [b]
Pulse wave velocity (m/s) Mean (SD)	5.77 (1.44)	5.65 (1.43)	5.94 (0.88)	**0.004[a]**

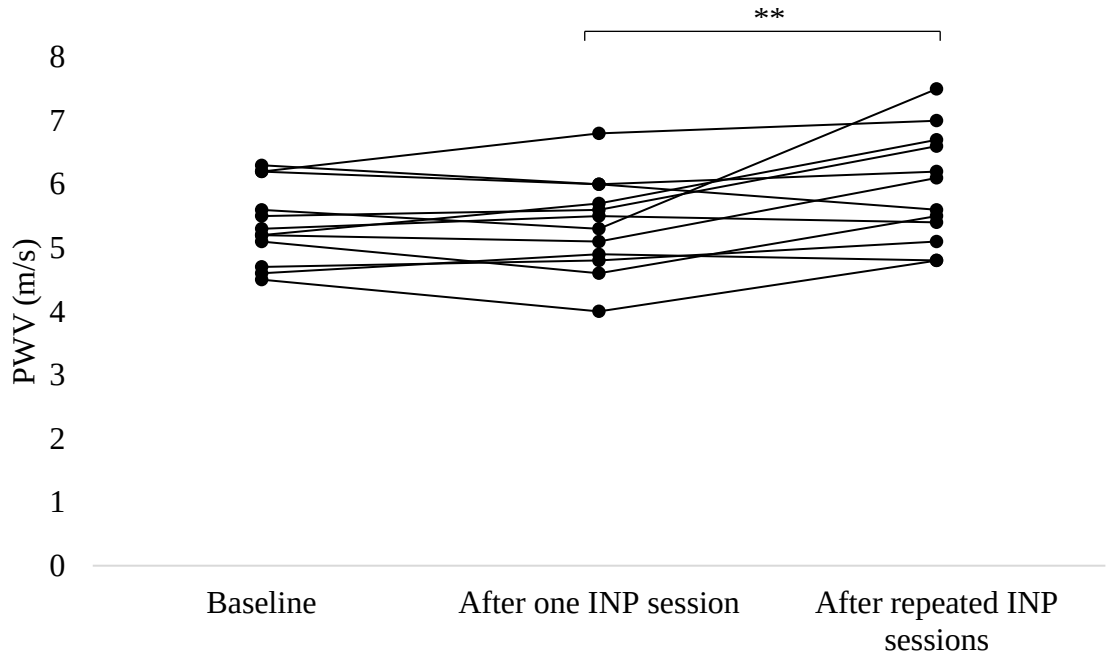

Figure 6.1.1 ***Pulse wave velocity measurements obtained in healthy volunteers at baseline, after one INP session and after repeated INP application.*** Data was compared using repeated measures ANOVA with post-hoc analysis. ** = $p < 0.01$. n = 12. PWV = pulse wave velocity, INP = intermittent negative pressure.

6.1.1.3 Endothelial function

Endothelial function was assessed in the dorsum of the foot of healthy volunteers using iontophoresis of vasoactive chemicals acetylcholine (ACh) and sodium nitroprusside (SNP). Baseline perfusion was measured for each iontophoresis chamber. Peak perfusion in response to each chemical was recorded and percentage change in response to ACh or SNP was calculated (Table 6.1.4). Baseline perfusion for both ACh and SNP were consistent between the three time points. Peak response to ACh did increase slightly after one INP session in comparison to baseline and after repeated INP however this was not statistically significant. Percentage change in perfusion in response to ACh increased from a median of 124% at baseline to 173% after one INP session and 165% after repeated INP however these differences were not statistically significant. Percentage change in perfusion in response to SNP increased after repeated INP compared to baseline and after one INP session however not significantly.

To assess the systemic effects of INP application, iontophoresis was performed on the forearm to assess cutaneous endothelial function at a site away from where INP was being applied. This was performed in 7 healthy volunteers (Table 6.1.5). Baseline perfusion in the ACh iontophoresis chamber was slightly higher after repeated INP sessions (median 56.20 versus 48.20 baseline and 48.60 after one INP session) however this was not statistically significant. While SNP baseline and peak perfusion remained relatively consistent between the three time points, percentage change in perfusion in response to SNP decreased from 111% at baseline to 66% after one INP session. After repeated INP, this figure rose to baseline levels at 120%. However, when compared using Friedman test, no significant changes were recorded.

Table 6.1.4: ***Endothelial function of the foot in healthy volunteers.*** Cutaneous endothelial function was assessed on the dorsum of the foot of healthy volunteers using iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP). Normally distributed data was compared using repeated measures ANOVA (a). Non-normally distributed data was compared using Friedman test (b). N = 12. ACh = acetylcholine, SNP = sodium nitroprusside, SD = standard deviation, IQR = interquartile range, AU = arbitrary units.

	Baseline	**After one INP session**	**After repeated INP sessions**	**p-value**
ACh Baseline Perfusion (au) Median (IQR)	23.60 (7.12)	20.85 (8.63)	23.65 (10.60)	0.338[b]
ACh Peak Perfusion (au) Median (IQR)	62.70 (30.77)	70.00 (45.80)	62.35 (39.15)	0.779[b]
ACh % Change Median (IQR)	124.00 (145.01)	173.38 (180.65)	165.60 (161.77)	0.264[b]
SNP Baseline Perfusion (au) Median (IQR)	23.35 (9.45)	21.45 (7.38)	21.60 (11.45)	0.144[b]
SNP Peak Perfusion (au) Mean (SD)	63.98 (23.08)	60.62 (26.21)	68.31 (24.56)	0.417[a]
SNP % Change Median (IQR)	147.97 (168.46)	144.69 (207.41)	182.95 (99.98)	0.472[b]

Table 6.1.5: ***Endothelial function of the forearm in healthy volunteers.*** Cutaneous endothelial function was assessed on the forearm in healthy volunteers using iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP). Normally distributed data was compared using repeated measures ANOVA (a). Non-normally distributed data was compared using Friedman test (b). n = 7. SD = standard deviation, IQR = interquartile range, AU = arbitrary units.

	Baseline	**After one INP session**	**After repeated INP sessions**	**p-value**
ACh Baseline Perfusion (au) Median (IQR)	48.20 (9.90)	48.60 (22.30)	56.20 (14.60)	0.156[b]
ACh Peak Perfusion (au) Mean (SD)	139.90 (40.52)	124.54 (45.50)	139.56 (42.72)	0.433[a]
ACh % Change Mean (SD)	153.78 (67.62)	141.21 (82.18)	147.83 (55.24)	0.881[a]
SNP Baseline Perfusion (au) Median (IQR)	51.70 (11.30)	53.00 (21.50)	48.70 (9.90)	0.565[b]
SNP Peak Perfusion (au) Mean (SD)	115.71 (22.37)	104.36 (45.94)	110.43 (34.84)	0.551[a]
SNP % Change Median (IQR)	111.03 (72.87)	66.71 (19.70)	120.12 (70.80)	0.368[b]

Correlation analysis was conducted between results obtained in the foot and in the forearm to assess whether endothelial function in both sites was related. A strong correlation between percentage change in perfusion in response to ACh in the arm versus the foot at baseline was observed upon Pearson correlation analysis however this was not statistically significant ($r = 0.619$, $p = 0.075$). A significant strong correlation was found between baseline ACh perfusion before INP application in the arm and foot ($r = 0.726$, $p = 0.027$). A strong correlation was observed for baseline ACh perfusion after one INP session in the arm and

foot however this was not significant (r = 0.600, p = 0.088). No other significant correlations were found between recordings obtained in the arm and foot.

6.1.1.4 Foot blood perfusion and skin temperature

Cutaneous blood perfusion was recorded using laser Doppler imaging in the dorsum of the foot of volunteers at baseline, after one INP session and after repeated INP sessions. This is different to the baseline perfusion recordings reported in Table 6.1.5 as the iontophoresis chambers refract the light causing inaccurate measurements for basal perfusion. In order to assess whether any changes in perfusion could be attributed to a change in skin temperature, skin temperature of the dorsum of the foot was recorded (Table 6.1.6). No significant differences in either foot blood perfusion or skin temperature were recorded between any of the time points in healthy volunteers.

Table 6.1.6: ***Foot blood perfusion and skin temperature in healthy volunteers.*** Cutaneous blood perfusion was measured in the dorsum of the foot of healthy volunteers at baseline, after one INP session and after repeated INP sessions. Data were compared using repeated measures ANOVA. N = 12. SD = standard deviation, au = arbitrary units.

	Baseline	**After one INP session**	**After repeated INP sessions**	**p-value**
Blood perfusion (au) Mean (SD)	24.50 (7.90)	21.86 (5.45)	23.53 (7.85)	0.274
Skin temperature (°C) Mean (SD)	31.07 (2.31)	30.39 (2.24)	30.83 (1.76)	0.518

6.1.2 Group 2: Comparison between baseline and after one INP application

As there were greater numbers of participants assessed at only two time points; baseline and after one INP session, analysis was completed between these two time points only to assess

significant changes which may have occurred. Baseline characteristics for 31 healthy volunteers who were assessed at baseline and after one INP session can be found in table 6.1.7. The median age of participants was 25 with ages ranging from 18 to 51. 10 males were recruited and 21 females. None of the participants were current cigarette smokers.

Table 6.1.7. ***Baseline characteristics for healthy volunteers.*** Baseline characteristics for healthy volunteers who were assessed at two-time points; baseline and after one INP session. IQR = interquartile range, SD = standard deviation, BMI = body mass index.

Total number (n)	31
Age years **Median (IQR)** **(Min – Max)**	25.0 (8.00) (18-51)
Sex	
Male n	10
Female n	21
Height cm **Median (IQR)**	167.00 (10.00)
Weight kg **Median (IQR)**	70.00 (20.00)
BMI kg/m^2 **Mean (SD)**	24.64 (4.07)

6.1.2.1 Central haemodynamics and skin temperature

Central haemodynamics were assessed at baseline and after one INP session (Table 6.1.8). A significant decrease in heart rate and diastolic blood pressure were observed after one INP session, versus baseline ($p = 0.026$, $p = 0.036$ respectively). However, systolic blood pressure did not significantly change. Skin temperature significantly decreased from a median of 31.40°C at baseline to 30.20°C after one INP session ($p = 0.024$).

Table 6.1.8: ***Skin temperature and central haemodynamics in healthy volunteers at baseline and post one INP session.*** Central haemodynamics measured were heart rate, systolic blood pressure and diastolic blood pressure. Measurements were taken at baseline and after one INP session. Normally distributed data was analysed with paired samples t-test. Non-normally distributed data was compared using Wilcoxon test. n = 31. SD = standard deviation, IQR = interquartile range, bpm = beats per minute.

	Baseline	**After one INP session**	**p-value**
Heart rate (bpm) Median (IQR)	63.00 (14.00)	61.00 (12.00)	**0.026**[b]
Systolic Blood Pressure (mmHg) Mean (SD)	120.13 (9.59)	118.06 (9.14)	0.096[a]
Diastolic blood pressure (mmHg) Median (IQR)	69.00 (13.00)	66.00 (12.00)	**0.036**[b]

6.1.2.2 Arterial stiffness

AIx significantly decreased from a baseline mean of 14.43 to 9.07 after one INP session (p=0.001) (Table 6.1.9). When adjusted for heart rate, this trend remained with AIx@75 decreasing from 9.90 at baseline to 2.37 post-INP (p = <0.001) (Figure 6.1.2). However, pulse wave velocity did not significantly change after one INP session when compared to baseline.

Table 6.1.9: ***Arterial stiffness measurements obtained from healthy volunteers at baseline and post one INP session.*** Arterial stiffness was assessed by measuring augmentation index (AIx), AIx adjusted for 75bpm and pulse wave velocity (PWV) at two different time points: baseline and after one INP session. Paired samples t-test was used to test normally distributed data (a) and Wilcoxon test for non-normally distributed data (b). AIx, AIx75 N = 30, PWV n = 29. AIx = augmentation index, PWV = pulse wave velocity, m/s = metres per second, SD = standard deviation, IQR = interquartile range.

	Baseline	**After one INP session**	**p-value**
Augmentation index (%AIx) Mean (SD)	14.43 (11.47)	9.07 (9.36)	**0.001[a]**
Augmentation index@75bpm (%AIx75) Mean (SD)	9.90 (10.82)	2.37 (10.08)	**2.3×10^{-5}[a]**
Pulse wave velocity (m/s) Median (IQR)	5.60 (1.60)	5.50 (1.65)	0.273[b]

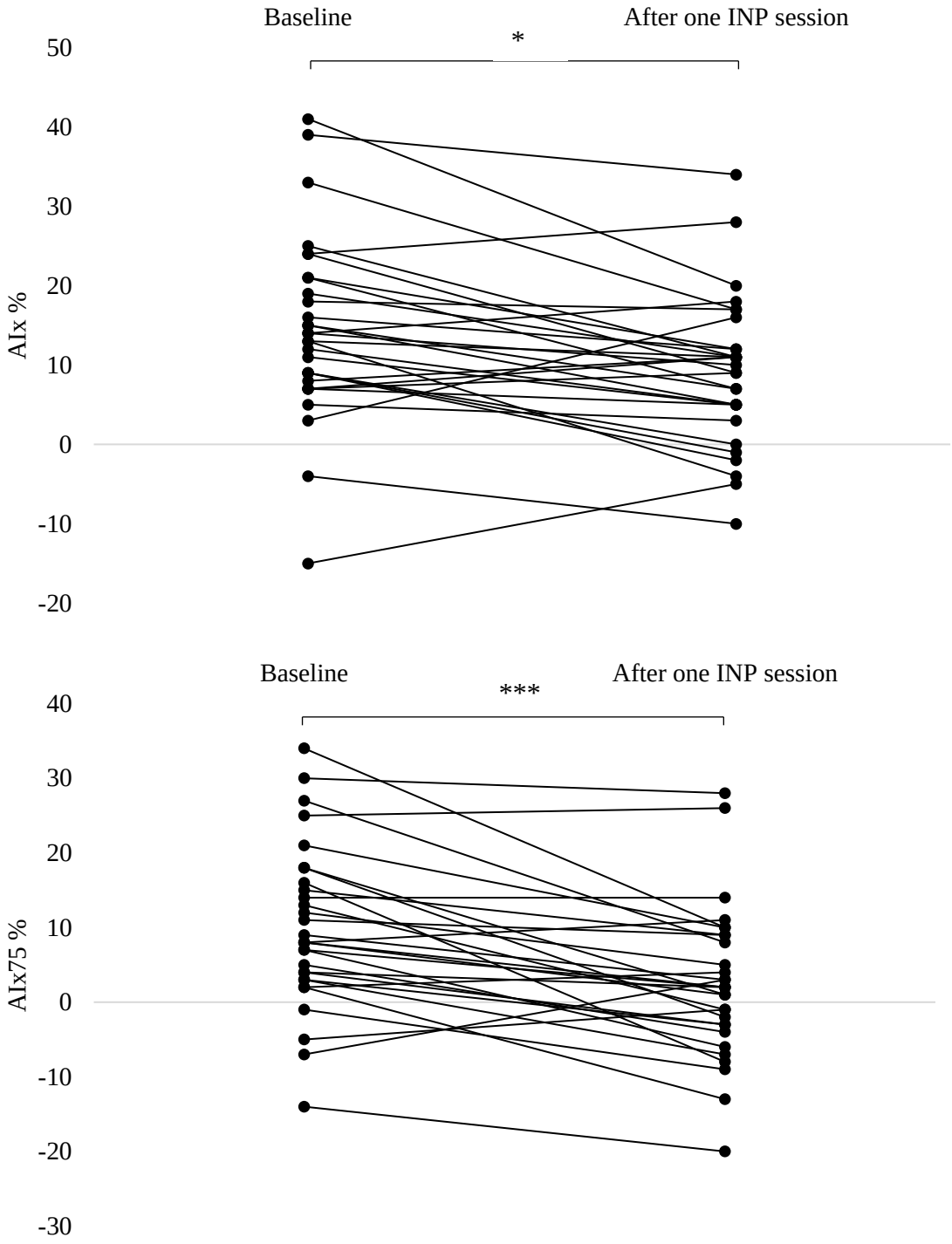

Figure 6.1.2 ***Augmentation index measurements obtained in healthy volunteers at baseline and after one INP session.*** Data was compared between time points using paired-samples t-test. * = $p < 0.05$, *** = $p < 0.001$. n = 30. AIx = augmentation index, AIx75 = augmentation index @ 75 beats per minute, INP = intermittent negative pressure.

6.1.2.3 Endothelial function

Endothelial function was assessed in the dorsum of the foot of healthy volunteers at baseline and after one INP session using the iontophoresis method previously described (Table 6.1.10). A significant difference in ACh baseline perfusion was observed after one INP session compared to baseline ($p = 0.013$). An increase in peak response and percentage change in perfusion was observed after one INP session. ACh percentage change increased from a median of 252% at baseline to 335% post-INP. However, neither of these changes were statistically significant when compared using Wilcoxon test. Baseline SNP perfusion remained consistent however peak perfusion significantly decreased after one INP session versus baseline ($p = 0.028$). As a result, percentage change in response to SNP decreased from 227% at baseline to 172% post-INP however this difference was not significant.

Table 6.1.10: ***Endothelial function of the foot in healthy volunteers at baseline and post one INP session.*** Cutaneous endothelial function was assessed on the dorsum of the foot of healthy volunteers using iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP). Data was non-normally distrusted and therefore compared using Wilcoxon signed ranks test. N = 31. ACh = acetylcholine, SNP = sodium nitroprusside, IQR = interquartile range, AU = arbitrary units.

	Baseline	**After one INP session**	**p-value**
ACh Baseline Perfusion (au) Median (IQR)	23.00 (10.80)	20.60 (10.20)	**0.013**
ACh Peak Perfusion (au) Median (IQR)	78.40 (65.20)	90.10 (39.30)	0.688
ACh % Change Median (IQR)	252.13 (296.89)	335.19 (325.29)	0.057
SNP Baseline Perfusion (au) Median (IQR)	22.70 (12.10)	22.50 (10.60)	0.433
SNP Peak Perfusion (au) Median (IQR)	78.60 (58.30)	62.60 (42.70)	**0.028**
SNP % Change Median (IQR)	227.75 (248.57)	172.39 (200.45)	0.544

6.1.2.4 Foot blood perfusion and skin temperature

As described previously, blood perfusion and skin temperature were measured in the dorsum of the foot before and after one INP session (Table 6.1.11). A significant decrease in blood perfusion was recorded in volunteers from a baseline mean of 24.70 to 22.70 post-INP (p =

0.001) (Figure 6.1.3). Skin temperature also decreased significantly from 31.40°C at baseline to 30.20°C after one INP session (p = 0.024).

Table 6.1.11: ***Foot blood perfusion and skin temperature in healthy volunteers at baseline and post one INP session.*** Cutaneous blood perfusion was measured in the dorsum of the foot of healthy volunteers at baseline and after one INP session. Data were compared using Wilcoxon signed ranks test. N = 31. IQR = interquartile range, au = arbitrary units.

	Baseline	**After one INP session**	**p-value**
Blood perfusion (au) Median (IQR)	24.70 (14.80)	22.70 (9.90)	**0.001**
Skin temperature (°C) Median (IQR)	31.40 (4.00)	30.20 (3.50)	**0.024**

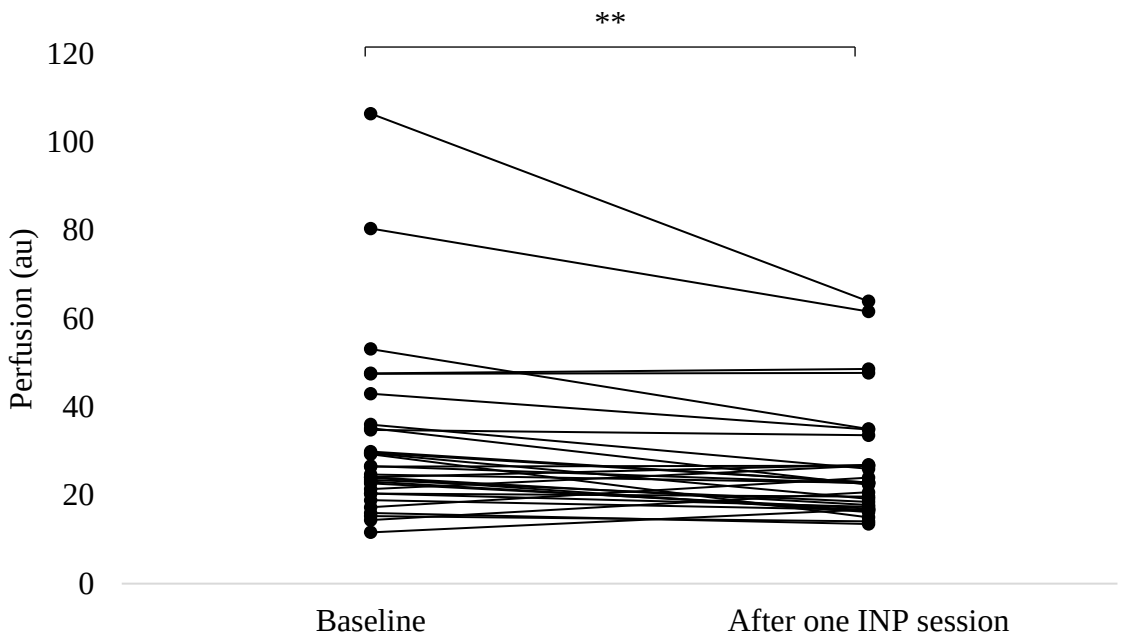

Figure 6.1.3 ***Foot cutaneous blood perfusion at baseline and after one INP session in healthy volunteers.*** Wilcoxon signed ranks test was used for data comparison. * = p < 0.05. n = 31. INP = intermittent negative pressure, au = arbitrary units.

6.1.3 One-hour INP

To determine effects of INP on blood perfusion while the negative pressure is applied laser imaging was used to scan the foot through the pressure chamber while INP was being applied.

During INP application, fluctuations in blood perfusion were observed in accordance with the negative pressure cycle (Figure 6.1.4). At the onset of negative pressure, there was a spike in blood perfusion which then fell slightly and remained consistent during the 10 seconds of negative pressure delivery. When the pressure was relived, there was a second spike in blood perfusion, larger than the first. This was followed by a fall back down to baseline levels.

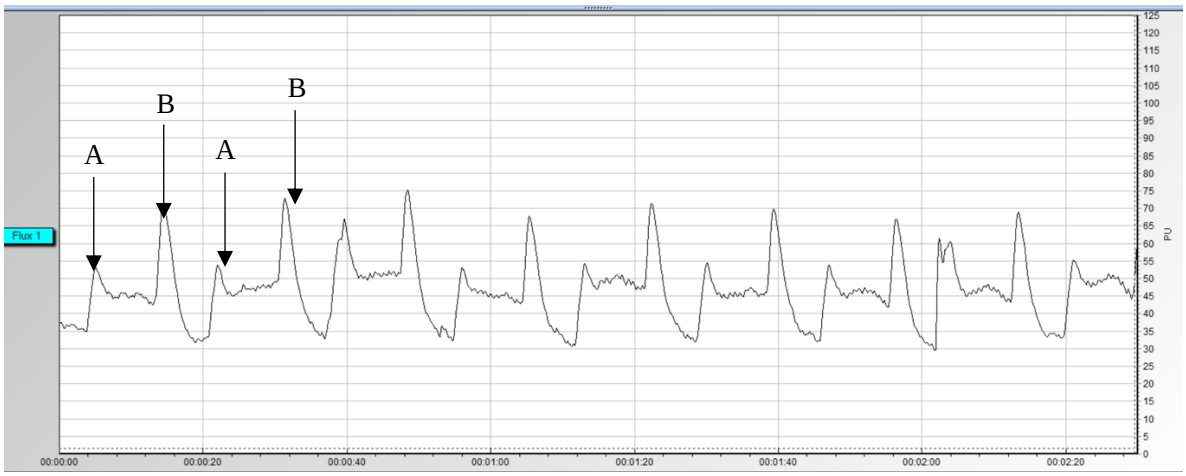

Figure 6.1.4 ***FLPI perfusion trace output during INP application to the foot.*** INP was applied to the foot of healthy volunteers and blood perfusion measured using FLPI. This trace depicts perfusion (PU) over time as INP is applied. Fluctuations in blood perfusion can be seen in line with the 10 second negative pressure and 7 second atmospheric pressure cycle. (A) Onset of negative pressure. (B) End of negative pressure, start of atmospheric pressure.

Cutaneous foot perfusion was assessed inside the pressure chamber, before and after INP application (Table 6.1.12). Similar to outside the pressure chamber (Table 6.1.11, Figure 6.1.3), blood perfusion decreased from 37.00 at baseline to 34.80 post-INP however, unlike outside the pressure chamber, this difference was not statistically significant ($p = 0.224$).

Table 6.1.12: ***Cutaneous blood perfusion in the dorsum of the foot of healthy volunteers before and after INP application inside the pressure chamber.*** Cutaneous blood perfusion of the dorsum of the foot was measuring using laser Doppler imaging, inside the pressure chamber, before and after one-hour INP application. Data was compared using Wilcoxon test (p = 0.224, n = 21). IQR = interquartile range, au = arbitrary units.

	Baseline	**After one INP session**	**p-value**
Perfusion (au) Median (IQR)	37.00 (28.25)	34.80 (33.05)	0.224

To assess if blood perfusion measurements outside and inside the pressure chamber were related, Spearman bivariant correlation analysis was conducted. Perfusion measured outside the pressure chamber at baseline was significantly correlated to perfusion measured inside the pressure chamber ($r = 0.840$, $p = 2x10^{-6}$). A significant strong correlation between perfusion inside and outside the pressure chamber was also recorded after INP application ($r = 0.625$, $p = 0.002$).

Blood perfusion measurements were taken inside the pressure chamber at five different time points to assess changes in foot blood perfusion while INP is being applied (Table 6.1.13) (Figure 6.1.5). The five time points were baseline (before INP was applied), at 10 minutes of INP delivery, at 30 minutes of INP, at 60 minutes of INP and post-INP delivery. Median perfusion increased during INP delivery compared to baseline and post-INP. Friedman test indicated a significant difference between the time points however after Wilcoxon signed ranks test with Bonferroni adjustment, no significant differences between any of the measurements was observed.

Table 6.1.13 ***Cutaneous blood perfusion in the dorsum of the foot of healthy volunteers inside the pressure chamber during INP application.*** Cutaneous blood perfusion of the dorsum of the foot was measured using laser Doppler imaging inside the pressure chamber at five different time points: before INP application, 10 minutes of INP application, 30 minutes INP application, 60 minutes INP application and post INP application. Friedman test with Wilcoxon signed rank post-hoc and Bonferroni adjustment was used to compare measurements. N = 21, $p = 9.8x10^{-8}$. IQR = interquartile range, au = arbitrary units.

	Baseline	**10 mins INP**	**30 mins INP**	**60 mins INP**	**Post INP**	**p-value**
Perfusion (au) Median (IQR)	37.00 (28.15)	49.10 (55.50)	47.40 (56.90)	44.80 (35.65)	34.80 (33.05)	< 0.001

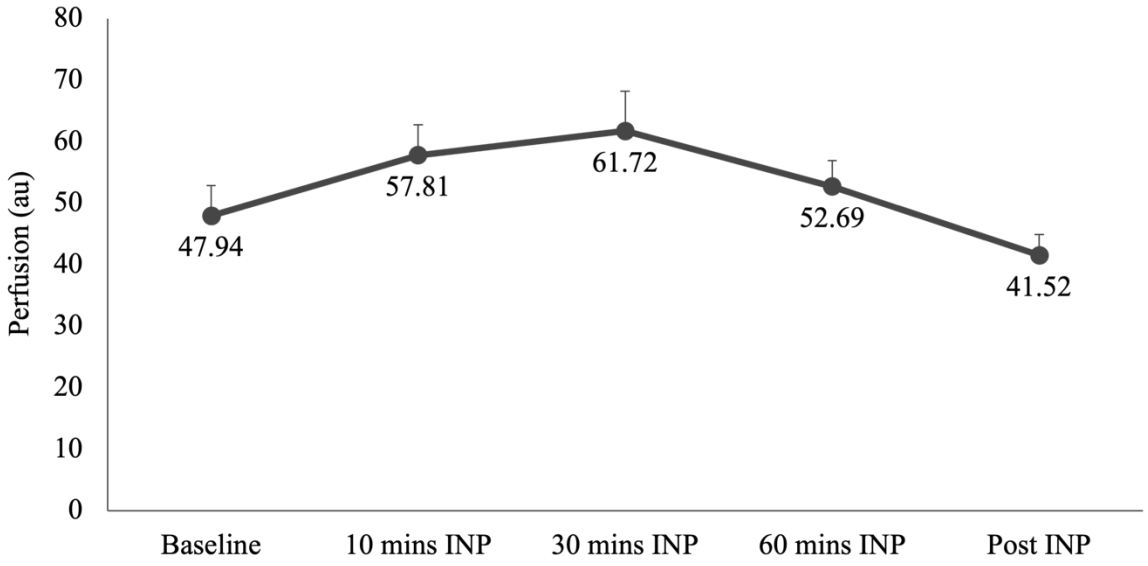

Figure 6.1.5 ***Foot blood perfusion before, during and after INP application in healthy volunteers.*** Data is presented as mean (+/- SEM). Friedman test and Wilcoxon signed rank post-hoc analysis with Bonferroni adjustment was used for statistical analysis of data. N = 21. Au = arbitrary units, INP = intermittent negative pressure, mins = minutes, SEM = standard error of the mean.

As the age range of healthy volunteers ranged from 18 to 51, Spearman correlation analysis was carried out between all measurements obtained and age. A significant correlation was found between age and percentage change in perfusion with ACh at baseline (n = 31, r = -0.436, p = 0.014). No significant correlations were found between age and ACh percentage change in perfusion at either of the other two time points (after one INP session and after

repeated INP sessions). No correlations were found between age and AIx or AIx75 however a significant correlation was found between age and PWV after one INP session ($n = 29$, $r = 0.443$, $p = 0.016$).

6.1.4 Group 3: Active versus Placebo INP

A placebo INP device was used in healthy volunteers to compare against the active device. 10 healthy volunteers used both the active and placebo devices, making the groups directly comparable. Volunteers were first tested with the placebo device on day one then retested using the active device between 2 and 5 days later to allow for enough time for any effects from the placebo device to no longer be present and a short enough time for cofounding variables to be minimised. Volunteer characteristics can be found in table 6.1.14. The mean age of participants was 24 years old, and ages ranged from 19 to 30 years old. 5 males and 5 females were recruited. There were no current smokers included and no participants reported being a previous smoker. All participants refrained from consuming caffeine for at least 4 hours before assessment.

Table 6.1.14: ***Baseline characteristics for healthy volunteers in active and placebo groups.*** Baseline characteristics for healthy volunteers who were assessed with both the active and placebo devices at two separate time points. SD = standard deviation, IQR = interquartile range.

Total number (n)	10
Age (years) **Mean (SD)** **(Min-max)**	24.2 (3.16) (19-30)
Sex	
Male	5
Female	5
Height (cm) **Mean (SD)**	170.90 (5.17)
Weight (kg) **Median (IQR)**	62.00 (23)
Body mass index (kg/m^2) **Mean (SD)**	22.68 (2.83)

6.1.4.1 Comparison between active and placebo INP

All measurements obtained from healthy volunteers in the active and placebo groups were compared (Table 5.1.15). No significant differences in skin temperature, blood pressure or heart rate were recorded between the two groups either before or after one session of INP. AIx and AIx75 were both lower at baseline in the placebo group than the active group however these differences were not significant. The opposite was true post-INP with median AIx and AxI75 being lower in the active group than the placebo group. No significant differences in PWV were observed between the two groups for either of the time points. A significant difference in peak perfusion in response to ACh was present between groups after one session of INP (active group mean = 92.04, placebo group mean = 164.91) ($p = 0.002$). ACh percentage change in perfusion was significantly higher in the placebo group at baseline (active mean 314%, placebo mean 483%) ($p = 0.032$). Although percentage change was also higher in the placebo group after INP application (active mean = 382%, placebo mean 564%),

this was not statistically significant when compared to the active group (p = 0.079). A significant difference in baseline SNP perfusion after one INP session was observed between groups (p = 0.013) however no significant differences between groups were recorded for SNP peak perfusion or SNP percentage change in perfusion before or after INP.

Table 6.1.15: ***Comparison between active and placebo healthy volunteer groups for all measurements.*** To assess whether any differences occurred between the two groups (active and placebo), all assessments were compared. Normally distributed data was compared with an independent samples t-test (a). Non-normally distributed data was compared using Mann-Whitney U test (b). Active N = 10, Placebo N = 10. Within group analysis comparing time points was also conducted (* = p < 0.05, ** = p < 0.01, *** p = <0.001). SD = standard deviation, IQR = interquartile range, bpm = beats per minute, AIx = augmentation index, ACh = acetylcholine, SNP = sodium nitroprusside, AU = arbitrary units.

		Active	**Placebo**	**P Value**
Skin Temperature (ºC)	**Before Median (IQR)**	32.00 (2.95)	31.40 (2.80)	0.543[b]
	After Mean (SD)	31.32 (2.77)	30.32 (1.90)	0.508[a]
Systolic Blood Pressure (mmHg)	**Before Median (IQR)**	120.50 (15)	116.00 (16)	0.449[b]
	After Mean (SD)	119.78 (10.23)	117.89 (9.45)	0.727[a]
Diastolic Blood Pressure (mmHg)	**Before Mean (SD)**	73.67 (8.97)	71.22 (7.93)	0.513[a]
	After Mean (SD)	69.00 (8.72)*	69.44 (9.66)	0.782[a]
Heart Rate (bpm)	**Before Mean (SD)**	65.67 (10.95)	67.00 (20.39)	0.935[a]
	After Mean (SD)	63.11 (16.00)	60.89 (18.65)**	0.851[a]
Augmentation Index (%AIx)	**Before Median (IQR)**	14.50 (12.50)	10.00 (17.00)	0.436[b]
	After Median (IQR)	7.00 (15.00)	10.00 (14.00)	0.343[b]

AIx75 (%)	**Before Mean (SD)**	5.67 (8.79)	2.89 (9.02)	0.438[a]
	After Median (IQR)	0.00 (13.75)**	2.50 (7.75)	0.596[b]
Pulse Wave Velocity (PWV) (ms^{-1})	**Before Mean (SD)**	6.47 (1.16)	6.62 (0.85)	0.749[a]
	After Mean (SD)	6.07 (1.13)	6.30 (0.77)*	0.615[a]
ACh Baseline Perfusion (au)	**Before Mean (SD)**	25.79 (5.13)	25.87 (8.13)	0.979[a]
	After Mean (SD)	20.39 (6.48)**	26.54 (8.40)	0.083[a]
ACh Peak Perfusion (au)	**Before Mean (SD)**	106.66 (54.29)	148.52 (42.10)	0.070[a]
	After Mean (SD)	92.04 (33.67)	164.91 (52.55)	**0.002[a]**
ACh % Change in Perfusion	**Before Mean (SD)**	314.25 (185.68)	483.25 (136.33)	**0.032[a]**
	After Mean (SD)	382.36 (189.38)	564.46 (244.16)	0.079[a]
SNP Baseline Perfusion (au)	**Before Median (IQR)**	25.75 (10.97)	22.20 (10.55)	0.406[b]
	After Median (IQR)	19.10 (15.10)*	31.35 (14.92)	**0.013[b]**
SNP Peak Perfusion (au)	**Before Mean (SD)**	103.74 (57.23)	130.28 (41.26)	0.250[a]
	After Mean (SD)	90.19 (58.38)	136.84 (51.97)	0.075[a]
SNP % Change in Perfusion	**Before Mean (SD)**	282.25 (163.70)	421.39 (171.35)	0.080[a]
	After Median (IQR)	303.34 (517.45)	250.27 (334.24)	0.705[b]

6.1.4.2 Placebo one-hour INP

As described in healthy volunteers with the active -40mmHg device (Section 6.1.3, Figure 6.1.4) fluctuations in blood perfusion were recorded in accordance with the negative pressure cycle as placebo INP was applied (Figure 6.1.6). Similar to the trace recorded with active INP, placebo INP caused fluctuations in perfusion in accordance with the negative pressure cycle with perfusion increasing slightly at negative pressure onset followed by a decrease. When negative pressure was relived, there was a second spike in perfusion however, unlike observed with active INP, this second spike was not greater than the first.

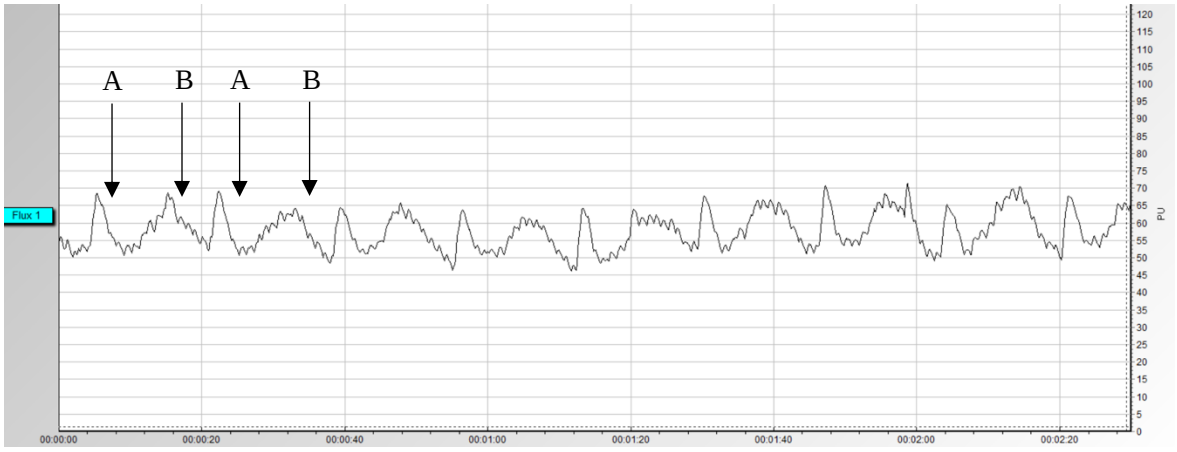

Figure 6.1.6 ***FLPI perfusion trace output during placebo INP application to the foot in healthy volunteers.*** Placebo INP was applied to the foot of healthy volunteers and blood perfusion measured using FLPI. This trace depicts perfusion (PU) over time as INP is applied. Blood perfusion increased upon application of negative pressure followed by a drop to baseline levels. After 10 seconds of negative pressure application, a rise in perfusion is recorded as pressure is relieved and a fall in perfusion during the 7 seconds of no pressure application. (A) Onset of negative pressure. (B) End of negative pressure and start of atmospheric pressure.

Cutaneous blood perfusion in the dorsum of the foot was recorded at baseline and after one placebo INP session outside of the pressure chamber (Table 6.1.16). A significant decrease in blood perfusion was recorded after one INP session (mean 30.30) compared to baseline (mean 38.21) (p=0.046). Foot blood perfusion was also assessed inside the pressure chamber, before and after placebo INP application (Table 6.1.17). Blood perfusion significantly decreased from a baseline mean of 49.63 to 39.36 post-INP (p=0.029).

As described previous, foot blood perfusion was measured inside the pressure chamber at five different time points then compared using a Friedman test (Table 6.1.18) (Figure 6.1.7). A significant difference between the time points was recorded (p=0.001) however after Wilcoxon signed ranks test with Bonferroni adjustment, no significant differences between any of the time points was reported.

Table 6.1.16: ***Cutaneous blood perfusion in the dorsum of the foot of healthy volunteers before and after placebo INP application outside the pressure chamber.*** Cutaneous blood perfusion of the dorsum of the foot was measuring using laser Doppler imaging, outside the pressure chamber, before and after one-hour placebo INP application. Data was compared using paired samples t-test (p = 0.046, n = 10). SD = standard deviation, AU = arbitrary units.

	Baseline	**After one INP session**	**p-value**
Perfusion (au) Mean (SD)	38.21 (19.92)	30.30 (15.78)	**0.046**

Table 6.1.17: ***Cutaneous blood perfusion in the dorsum of the foot of healthy volunteers before and after placebo INP application inside the pressure chamber.*** Cutaneous blood perfusion of the dorsum of the foot was measuring using laser Doppler imaging, inside the pressure chamber, before and after one-hour placebo INP application. Data was compared using paired samples t-test (p = 0.029, n = 10). SD = standard deviation, AU = arbitrary units.

	Baseline	**After one INP session**	**p-value**
Perfusion (au) Mean (SD)	49.63 (26.95)	39.36 (21.22)	**0.029**

Table 6.1.18: ***Cutaneous blood perfusion in the dorsum of the foot of healthy volunteers inside the pressure chamber during placebo INP application.*** Cutaneous blood perfusion of the dorsum of the foot was measured using laser Doppler imaging inside the pressure chamber at five different time points: before INP application, 10 minutes of INP application, 30 minutes INP application, 60 minutes INP application and post INP application. Friedman test combined with post-hoc Wilcoxon analysis and Bonferroni adjustment was conducted (p = 0.001, n = 10). Post hoc analysis revealed no significant differences between any of the time points.. IQR = interquartile range.

	Baseline	**10 mins INP**	**30 mins INP**	**60 mins INP**	**Post INP**	**p-value**
Perfusion (au) Median (IQR)	46.30 (41.97)	49.60 (46.10)	48.60 (47.88)	41.95 (35.58)	33.70 (24.15)	0.001

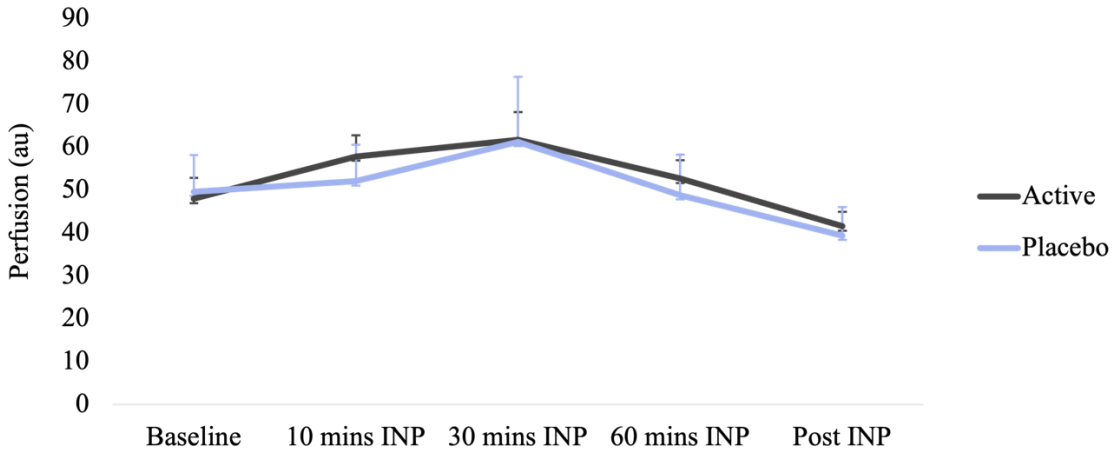

Figure 6.1.7 ***Foot blood perfusion before, during and after active and placebo INP application in healthy volunteers.*** Data is presented as mean (+/- SEM). Friedman test and Wilcoxon signed rank post-hoc analysis with Bonferroni adjustment was used for statistical analysis of data. N = 10. Au = arbitrary units, INP = intermittent negative pressure, mins = minutes, SEM = standard error of the mean.

6.1.4.2.1 Comparison between active and placebo one-hour INP

Perfusion measurements obtain outside the pressure chamber before INP application and inside the pressure chamber at the five different time points were compared between groups (Table 6.1.19). No significant differences in perfusion were recorded between groups. For all

three time points while INP was being applied (10 mins, 30 mins and 60 mins) blood perfusion was higher in the active group than the placebo however these differences were not significant.

Table 6.1.19: ***Comparison between active and placebo group for foot blood perfusion before, during and after INP application.*** Blood perfusion measurements collection in both active and placebo groups were compared. Normally distributed data was compared using independent samples t-test (a) and non-normally distributed data was analysed using Mann-Whitney U test (b). Active n = 10, placebo n = 10. SD = standard deviation, IQR = interquartile range, au = arbitrary units.

		Active	**Placebo**	**P value**
Perfusion (au)	**Outside Baseline Mean (SD)**	36.55 (18.49)	38.21 (19.92)	0.849[a]
	Outside After one INP session Mean (SD)	30.57 (16.65)	30.30 (15.78)	0.971[a]
	Inside Baseline Mean (SD)	52.80 (30.48)	49.64 (26.95)	0.808[a]
	10 mins INP (Median (IQR)	53.81 (51.73)	49.60 (46.10)	0.393[b]
	30 mins INP Median (IQR)	69.05 (63.23)	48.60 (47.88)	0.353[b]
	60 mins INP Median (IQR)	64.75 (38.63)	41.95 (35.58)	0.105[b]
	Inside After one INP session Mean (SD)	48.40 (19.80)	39.36 (21.22)	0.338[a]

6.1.5 Wavelet Analysis

Wavelet analysis was carried out on the FLPI perfusion traces collected from healthy volunteer's feet at baseline, during INP and after one INP session. Percentage contribution of each of the six components (cardiac, respiratory, myogenic, sympathetic, endothelial, and non-endothelial) was recorded for participants who used the active device and placebo device.

When comparing before and after INP application, no significant differences in percentage contribution of any of the six components was recorded in either the active or placebo group except for the cardiac component in the active group, which significantly decreased from a baseline mean of 1.25% or 1.05% post-INP ($p=0.046$). In both groups at both time points, the greatest contribution was from the endothelial component. When comparing between groups, no significant differences for any of the components at either time point was recorded (Table 6.1.20). During INP application, the highest contributing component in the active group was the myogenic component across all three time points. In the placebo group, the highest contributing component at 10 minutes INP was myogenic (median of 26.59%), at 30 and 60 minutes INP was the endothelial component (median of 28.18% and 26.57% respectively). When comparing each component at each time point between groups, several significant differences were recorded (Table 6.1.21). The respiratory and myogenic components contributed significantly higher in the active group than the placebo group at all three time points while the sympathetic component contributed higher in the placebo group than the active group across all three time points (Figure 6.1.8). The endothelial component contributed significantly higher at 30 and 60 minutes of INP application in the placebo group versus the active group (Figure 6.1.8). No significant differences in cardiac or non-endothelial component contributions were recorded between groups for any of the three time points (Figure 6.1.8).

Table 6.1.20: ***Between group comparisons for each component before and after INP application.*** Measurements obtained from healthy volunteers at baseline and after one active or placebo INP session were compared. Normally distributed data was compared using independent samples t-test (a). Non-normally distributed data was compared using Mann-Whitney U test (b). n = 30. IQR = interquartile range, SD = standard deviation.

		Active	Placebo	p value
Cardiac (%)	**Before Mean (SD)**	1.25 (0.47)	1.44 (0.44)	0.302[a]
	After Mean (SD)	1.05 (0.43)	1.18 (0.33)	0.395[a]
Respiratory (%)	**Before Mean (SD)**	5.95 (2.75)	6.11 (2.95)	0.881[a]
	After Mean (SD)	5.21 (2.40)	4.24 (1.06)	0.135[a]
Myogenic (%)	**Before Mean (SD)**	13.94 (4.48)	16.51 (5.50)	0.181[a]
	After Mean (SD)	13.75 (4.36)	13.36 (3.62)	0.809[a]
Sympathetic (%)	**Before Mean (SD)**	22.42 (3.59)	22.89 (5.59)	0.824[a]
	After Mean (SD)	23.99 (5.66)	22.62 (4.78)	0.516[a]
Endothelial (%)	**Before Median (IQR)**	33.21 (5.80)	31.08 (3.34)	0.546[b]
	After Mean (SD)	34.40 (7.48)	34.01 (6.45)	0.888[a]
Non-endothelial (%)	**Before Mean (SD)**	23.82 (8.02)	21.74 (9.96)	0.541[a]
	After Mean (SD)	21.60 (6.97)	24.59 (8.18)	0.304[a]

Table 6.1.21. ***Between group comparisons for each component during active and placebo INP application.*** Measurements obtained from healthy volunteers during active and placebo INP application were compared. Normally distributed data was compared using independent samples t-test (a). Non-normally distributed data was compared using Mann-Whitney U test (b). n = 30. IQR = interquartile range, SD = standard deviation.

		Active	Placebo	p value
Cardiac (%)	**10 mins Mean (SD)**	1.42 (0.29)	1.58 (0.58)	0.315[a]
	30 mins Mean (SD)	1.36 (0.45)	1.47 (0.52)	0.536[a]
	60 mins Mean (SD)	1.38 (0.45)	1.39 (0.41)	0.970[a]
Respiratory (%)	**10 mins Mean (SD)**	17.83 (3.75)	12.39 (4.65)	**0.002[a]**
	30 mins Mean (SD)	17.62 (6.09)	11.22 (3.56)	**0.001[a]**
	60 mins Mean (SD)	17.36 (5.40)	11.37 (3.71)	**0.004[a]**
Myogenic (%)	**10 mins Mean (SD)**	33.90 (5.38)	28.06 (5.76)	**0.011[a]**
	30 mins Mean (SD)	33.32 (7.83)	24.72 (4.23)	**0.001[a]**
	60 mins Median (IQR)	31.81 (8.05)	22.11 (6.34)	**0.001[b]**
Sympathetic (%)	**10 mins Mean (SD)**	14.32 (2.81)	19.47 (2.80)	**0.000058[a]**
	30 mins Mean (SD)	14.77 (3.71)	19.52 (3.33)	**0.002[a]**
	60 mins Mean (SD)	14.84 (2.78)	18.20 (3.04)	**0.005[a]**
Endothelial (%)	**10 mins Mean (SD)**	18.56 (4.82)	22.84 (6.65)	0.053[a]
	30 mins Mean (SD)	20.38 (5.62)	28.18 (5.13)	**0.001[a]**
	60 mins Mean (SD)	20.28 (4.41)	26.57 (3.53)	**0.001[a]**
Non-endothelial (%)	**10 mins Mean (SD)**	13.97 (5.12)	15.66 (7.97)	0.487[a]

	30 mins Median (IQR)	11.17 (8.58)	13.10 (5.88)	0.286[b]
	60 mins Mean (SD)	14.15 (7.48)	18.30 (4.36)	0.118[a]

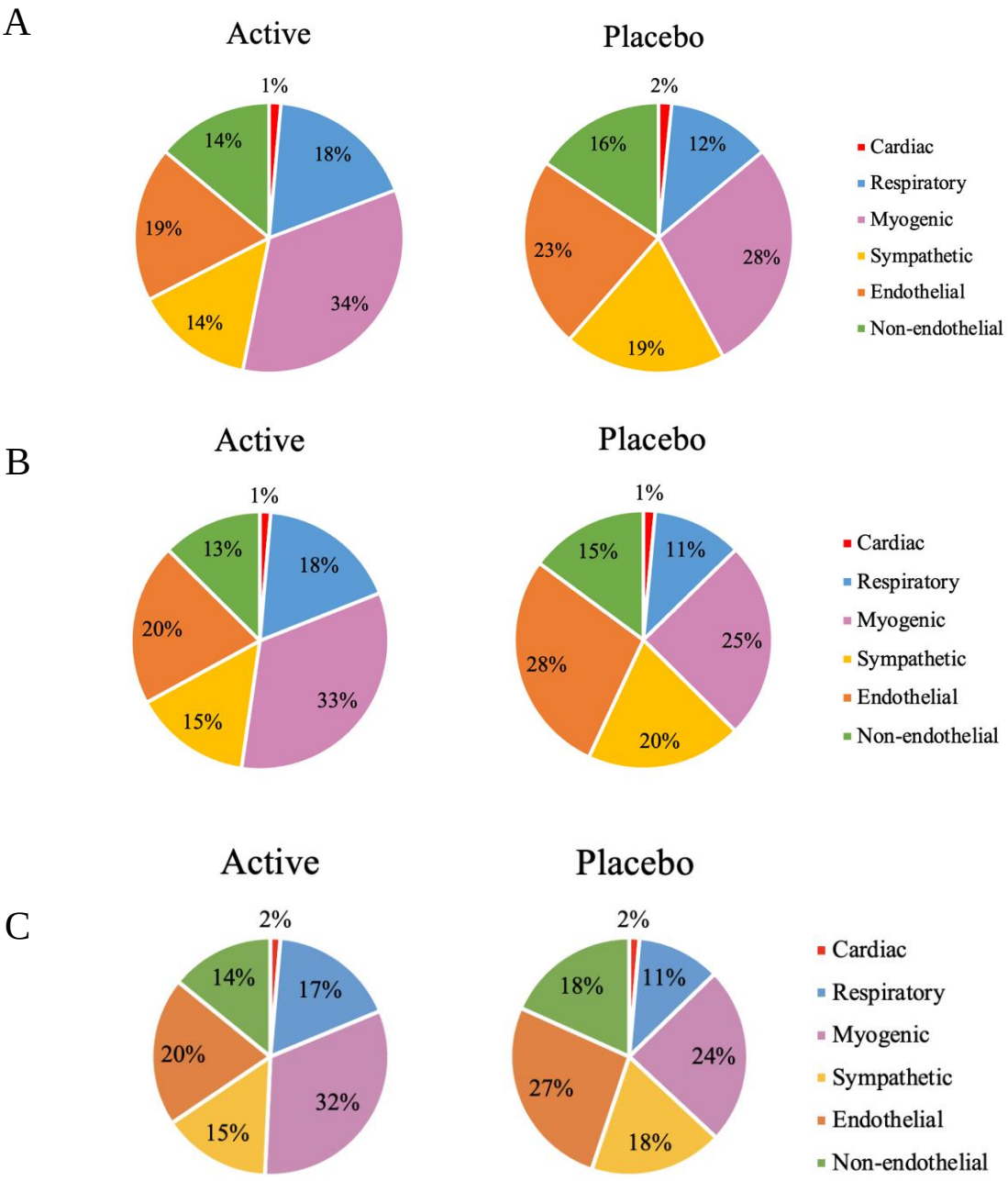

Figure 6.1.8 ***Contribution of each regulatory component to perfusion during active or placebo INP application in healthy volunteers.*** The percentage contribution of each regulatory component to the overall perfusion signal is presented here at different time points during active or placebo INP application. (A) 10 minutes INP, (B) 30 minutes INP and (C) 60 minutes INP. N = 30. INP = intermittent negative pressure.

6.1.6 Blood inflammatory and oxidative stress biomarkers

Blood samples collected from healthy volunteers were tested for blood biomarkers of inflammation and oxidative stress namely ICAM-1, MMP-8, VCAM-1, IL-1a, E-selectin and MPO in order to evaluate the systemic effects of repeated INP application (Table 6.1.22). In healthy individuals, levels of all measured biomarkers did not significantly change after repeated INP application, compared to baseline.

Table 6.1.22 ***Blood borne inflammatory and oxidative stress biomarkers at baseline and after repeated INP in healthy volunteers.*** Inflammatory and oxidative stress markers ICAM-1, MMP-8, VCAM-1, IL-1a, E-selectin and MPO were measured in serum and plasma samples obtained from healthy volunteers at baseline and after repeated INP application for 5 consecutive days. Normally distributed data was compared using paired-samples t-test (a). Non-normally distributed data was analysed using Wilcoxon signed rank test (b). n = 9. ICAM-1 = intercellular adhesion molecule 1, MMP-8 = matrix metalloproteinase 8, VCAM-1 = vascular cell adhesion protein 1, IL-1a = interleukin-1 alpha, E-selectin = endothelial-selectin, MPO = myeloperoxidase, IQR = interquartile range, SD = standard deviation.

	Baseline	**Post repeated INP**	**p-value**
ICAM-1 (ug/ml) Median (IQR)	1.10 (0.21)	1.08 (0.17)	0.441[b]
MMP-8 (ug/ml) Mean (SD)	$8.1x10^{-3}(1.8x10^{-3})$	$8.6x10^{-3}$ $(1.6x10^{-3})$	0.370[a]
VCAM-1 (ug/ml) Median (IQR)	1.53 (0.22)	1.37 (0.48)	0.214[b]
IL-1a (ug/ml) Mean (SD)	$1.9x10^{-4}$ $(7.2x10^{-5})$	$1.8x10^{-4}$ $(3.6x10^{-5}$ 36.15)	0.818[a]
E-selectin (ug/ml) Mean (SD)	0.08 (0.02)	0.08 (0.01)	0.279[a]
MPO (ug/ml) Median (IQR)	0.26 (0.30)	0.26 (0.29)	0.314[b]

6.1.7 Summary of findings

A total of 31 healthy volunteers were recruited to the study. 12 healthy volunteers were tested at three time points; baseline, after one INP session and after repeated INP sessions and 31 volunteers were tested at two time points; baseline and after one INP session.

- AIx and AIx75 significantly decreased after one INP session, compared to baseline.
- Compared to baseline, no changes in endothelial function were observed after a single bout of INP or after repeated INP sessions.
- INP application to the foot of healthy volunteers had no effect on systemic microvascular endothelial function.
- Foot blood perfusion and skin temperature both significantly decreased after one INP session
- There were no differences in perfusion or vascular function assessments between the active and placebo devices
- No significant changes in contribution of any of the six vasomotion components was recorded between time points.
- Significant differences in respiratory, myogenic, sympathetic, and endothelial components contributions to overall perfusion were recorded between active INP and placebo INP.
- No changes in levels of blood borne inflammatory or oxidative stress markers was recorded after repeated INP application, versus baseline.

6.2 Foot study: PAD patients

Table 6.2.1: ***Baseline patient characteristics.*** Characteristics for recruited patients at baseline. SD = standard deviation, BMI = body mass index, T1DM.= type 1 diabetes mellitus, T2DM = type 2 diabetes mellitus.

N	21
Sex (Male/Female)	16/5
PAD Status (tissue loss/no tissue loss)	4/17
Age (years) **Mean (SD)** **Min - max**	70.8 (6.27) 60 - 85
Height (cm) **Mean (SD)**	169.67 (7.98)
Weight (kg) **Mean (SD)**	84.71 (21.53)
BMI (kg/m^2) **Mean (SD)**	29.07 (6.05)
Smokers	
Current Smoker (n)	5
Previous Smoker (n)	10
Never (n)	6
Diabetes	
T1DM (n)	2
T2DM (n)	9
Non-diabetic (n)	10

To determine the more prolonged effects of INP on vascular function and pain, patients used the device at home for two hours per day for between four to eight weeks. Assessments were made at baseline, after a one-hour INP session and after repeated INP sessions at home. Only 5 of the 21 patients were assessed at all three time points. 11 patients were assessed at baseline and after one INP session and 14 participants were assessed at baseline and after repeated INP. Average compliance, as recorded by the Flow-Ox device, was 71%. Patients whose compliance was below 50% were excluded. No patients were excluded based on poor compliance.

Baseline patient characteristics can be found in table 6.2.1. 21 patient's data were included in the data analysis. 16 males and 5 females were recruited. 17 patients exhibited rest pain with no tissue loss while 4 patients exhibited tissue loss in the form of ulcer(s) and/or gangrene. Mean age was 70.81 with the youngest being 60 years old and eldest 85 years old. Five patients reported being current smokers, 10 reported having been previous smokers and six had never smoked. 10 of the 21 patients did not suffer from any form of diabetes. 2 patients exhibited type 1 diabetes mellites (T1DM) and nine suffered from type 2 diabetes mellitus (T2DM). Patients were on a variety of medication of their condition and several comorbidities including diabetes, hypertension, high cholesterol, and chronic obstructive pulmonary disease (COPD). These medications included aspirin, clopidogrel, ACE inhibitors, metformin, insulin, and statins. All patients were of white ethnicity. No safety concerns with regards to use of the Flow-Ox device arose during the study with no side effects or adverse events being reported in these patients.

6.2.1 Comparison between baseline, one INP session and repeated INP

Five patients were assessed at all three time points: baseline, after one INP session and after repeated INP sessions. Their assessments across the three time points are described within this section.

6.2.1.1 Central haemodynamics

Central haemodynamics were assessed in patients by measurement of systolic blood pressure, diastolic blood pressure and heart rate (Table 6.2.2). Blood pressure remained consistent at each of the time points. Heart rate was highest at baseline (median of 74bpm) compared to post one INP session and post repeated INP. Friedman test revealed a statistically significant difference in heart rate between the three time points ($p = 0.041$). Post-hoc analysis using Wilcoxon signed rank test with Bonferroni adjustment revealed a significant difference between baseline and post one INP session ($p=0.010$) but not between baseline and post-repeated INP or between post-one INP session and post-repeated INP.

Table 6.2.2: ***Recorded central haemodynamics in PAD patients at baseline, post one INP session and post repeated INP.*** Vital central haemodynamics measured were heart rate, systolic blood pressure and diastolic blood pressure. Measurements were taken at baseline, after one INP session and after repeated INP sessions. Normally distributed data was analysed with repeated measures ANOVA (a). Non-normally distributed data was analysed using Friedman test (b). Post hoc analysis was conducted with Wilcoxon singed rank test with Bonferroni adjustment. N = 5. SD = standard deviation, IQR = interquartile range, bpm = beats per minute.

	Baseline	**Post one INP session**	**Post Repeated INP**	**p-value**
Systolic blood pressure (mmHg) Mean (SD)	146.60 (18.34)	142.80 (17.85)	140.20 (21.57)	0.869[a]
Diastolic blood pressure (mmHg) Mean (SD)	72.80 (10.06)	74.20 (5.54)	71.20 (12.07)	0.870[a]
Heart rate (bpm) Median (IQR)	74.0 (18.0)	63.00 (18.50)	65.00 (14.50)	**0.041[b]**

6.2.1.2 Arterial stiffness

Arterial stiffness was assessed by measuring augmentation index (AIx), augmentation index adjusted for 75bpm (AIx75) and pulse wave velocity (PWV) (Table 6.2.3). No significant changes in AIx or AIx75 were recorded however AIx75 was lower post one INP session (median 24) compared to baseline (median 35) and post repeated INP (median 34). PWV increased after repeated INP to a mean of 8.36 m/s compared to a baseline mean of 7.50 m/s and post one INP session mean of 6.68m/s however these differences were not statically significant.

Table 6.2.3: ***Arterial stiffness measurements obtained from PAD patients at baseline, post one INP session and post repeated INP.*** Arterial stiffness was assessed by measuring augmentation index (AIx), AIx adjusted for 75bpm and pulse wave velocity (PWV) at the three different time points; baseline, after one INP session and after repeated INP sessions. Repeated measures ANOVA was used for normally distributed data (a) and Friedman test was used for non-normally distributed data (b). AIx n = 5, AIx75 n = 5, PWV n = 4. SD = standard deviation, IQR = interquartile range.

	Baseline	**Post one INP session**	**Post Repeated INP**	**p-value**
Augmentation index (AIx) Median (IQR)	36.00 (3.75)	35.00 (8.50)	40.00 (5.00)	0.247[b]
Augmentation index@75bpm Median (IQR)	35.00 (16.00)	24.00 (19.00)	34.00 (22.00)	0.143[b]
Pulse wave velocity (m/s) Mean (SD)	7.50 (1.78)	6.68 (3.79)	8.38 (1.79)	0.321[a]

6.2.1.3 Pain and ABPI

Patients rated their pain on a scale of 0 – 10 at baseline and post repeated INP. Pain score recorded by the patients significantly reduced after repeated INP application from a median of 8 at baseline to 6 post-INP (p=0.001) (Figure 6.2.1). Pain score reported improved in 13 out of 21 patients. Pain score remained the same in 6 cases. 2 patients reported a higher pain score after repeated INP application than at baseline. Ankle-brachial pressure index (ABPI) was measured in 11 patients at baseline and post repeated INP. ABPI measurement remained consistent before and after INP application (Table 6.2.4).

Table 6.2.4: ***Pain score and ABPI measurements obtained from PAD patients.*** Pain score (0-10) was reported by patients at baseline and post repeated INP. ABPI was measured in patients baseline and post repeated INP. Wilcoxon test was used to compare data at baseline and post repeated INP. Pain n = 21, ABPI n = 11. IQR = Interquartile range, ABPI = ankle-brachial pressure index.

	Baseline	**Post Repeated INP**	**p-value**
Pain Median (IQR)	8.00 (2.00)	6.00 (4.00)	**0.001**
ABPI Median (IQR)	0.55 (0.42)	0.52 (0.30)	0.168

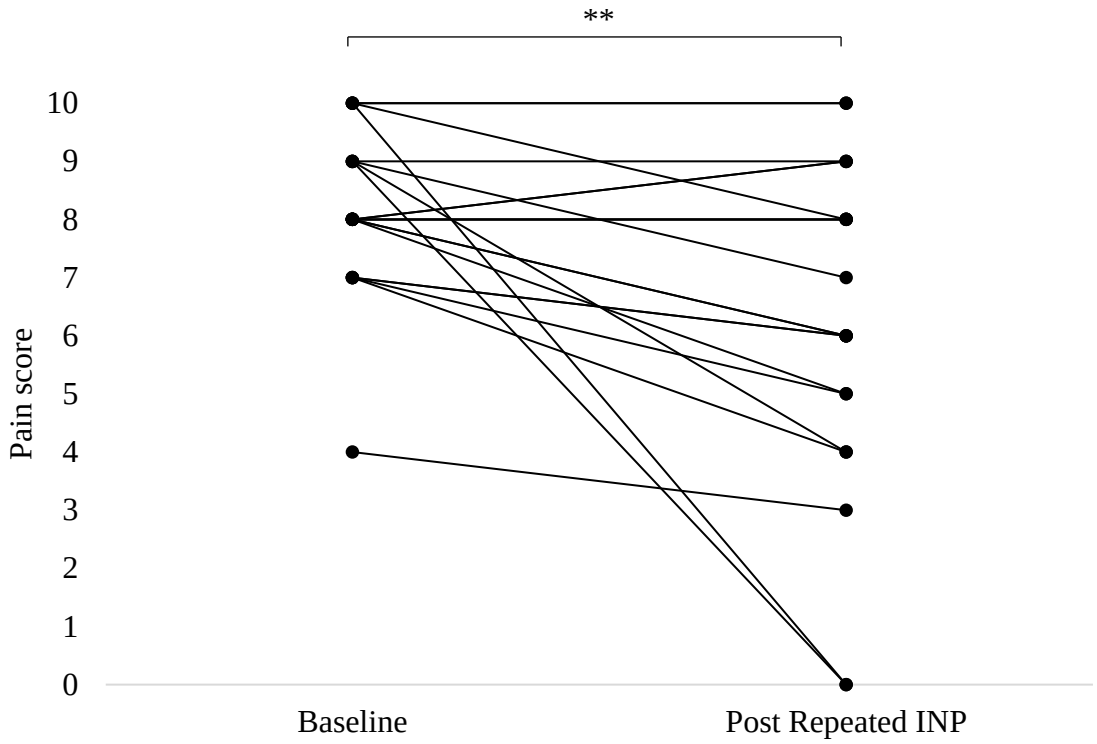

Figure 6.2.1 ***Pain score recorded by PAD patients before and after repeated INP application.*** Data were compared using Wilcoxon test. ** = $p < 0.01$. n = 21. INP = intermittent negative pressure.

6.2.1.4 Endothelial function

Endothelial function was assessed in the foot of patients using iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) at baseline, post one INP session and post repeated INP (Table 6.2.5). Baseline ACh perfusion remained consistent between each of the three

time points. Analysis revealed a significant difference in peak perfusion in response to ACh between the three time points ($p = 0.015$). However, post-hoc analysis revealed no significant differences between measurements obtained at baseline and after one INP session ($p = 0.088$), baseline and after repeated INP sessions ($p = 0.053$) or after one INP session and after repeated INP sessions ($p = 1.00$). ACh percentage change in perfusion was higher post one INP session (mean 199%) compared to baseline (mean 117%) and post repeated INP (mean 182%) however this was not statistically significant between measurements. SNP percentage change in perfusion increased post one INP session compared to baseline (median 102% and 64%, respectively). Post repeated INP application, SNP percentage change decreased compared to post one INP session but increased in comparison to baseline. However, upon statistical analysis, no statistical differences were observed between the three time points.

Table 6.2.5: ***Endothelial function of the foot in PAD patients at baseline, post one INP session and post repeated INP.*** Cutaneous endothelial function was assessed on the dorsum of the foot of PAD patients using iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP). Normally distributed data was compared using repeated measures ANOVA with post-hoc analysis as required (a). Non-normally distributed data was compared using Friedman test (b). n = 5. SD = standard deviation, IQR = interquartile range, AU = arbitrary units, ACh = acetylcholine, SNP = sodium nitroprusside.

	Baseline	**Post one INP session**	**Post Repeated INP**	**p-value**
Baseline ACh Perfusion (au) Mean (SD)	30.66 (6.92)	32.80 (30.90)	35.32 (9.92)	0.720[a]
Peak ACh Perfusion (au) Mean (SD)	64.74 (19.37)	91.60 (22.54)	97.80 (25.63)	**0.015[a]**
Percentage Change ACh (%) Mean (SD)	117.48 (78.51)	198.81 (98.57)	181.58 (59.35)	0.136[a]
Baseline SNP Perfusion (au) Mean (SD)	16.64 (12.29)	26.76 (5.06)	28.10 (1.28)	0.480[a]
Peak SNP Perfusion (au) Mean (SD)	66.04 (20.32)	66.88 (27.28)	68.68 (32.76)	0.982[a]
Percentage Change SNP (%) Median (IQR)	64.24 (186.88)	101.82 (246.71)	93.97 (153.69)	0.549[b]

6.2.1.5 Foot blood perfusion and skin temperature

Cutaneous blood perfusion was measured in the dorsum of the foot in patients at baseline, after one INP session and after repeated INP (Table 6.2.6). Although perfusion increased across the three time points; mean at baseline 44.82, mean after one INP session 54.60, mean after repeated INP 62.62, these differences were not statistically significant (p = 0.118). Skin

temperature was assessed in the dorsum of the foot of patients at each of the time points to determine if any changes in perfusion were related to skin temperature changes however no significant changes in skin temperature were recorded between any of the three time points.

Table 6.2.6: ***Cutaneous blood perfusion and skin temperature in the dorsum of the foot of PAD patients outside the pressure chamber.*** Cutaneous blood perfusion and skin temperature of the dorsum of the foot was measuring using laser Doppler imaging, outside of the pressure chamber at baseline, after one INP session and after repeated INP. Data was compared using repeated measures ANOVA. N = 5. SD = standard deviation, au = arbitrary units.

	Baseline	**Post one INP session**	**Post Repeated INP**	**p-value**
Perfusion (au) Mean (SD)	44.82 (13.66)	54.60 (19.02)	62.62 (14.53)	0.118
Skin temperature (°C) Mean (SD)	30.24 (0.83)	30.28 (1.90)	31.28 (0.83)	0.305

6.2.2 Comparison between baseline and one INP session

As a larger number of patients were assessed at two time points; baseline and after one INP session, assessments collected from patients at these two time points were compared against one another. 11 patients were assessed at the two time points: baseline and after one INP session (Table 6.2.7). 8 of these were male and 3 were females. Of these patients, 2 exhibited tissue loss and 9 did not exhibit tissue loss. The mean age of these patients was 69.91 years old. 3 patients had never previously been a smoker while 6 reported being previous smokers and 2 were current smokers. Of all the patients, 2 suffered from type 1 diabetes mellitus, 4 suffered from type 2 diabetes mellitus and 5 did not suffer from diabetes of any kind.

Table 6.2.7: ***Baseline characteristics for patients assessed at baseline and after one INP session.*** Baseline characteristics for patients who were assessed at tow time points: baseline and post one INP session. SD = standard deviation, BMI = body mass index, T1DM = type 1 diabetes mellitus, T2DM = type 2 diabetes mellitus, PAD = peripheral arterial disease.

N	11
Sex (Male/Female)	8/3
PAD Status (tissue loss/no tissue loss)	2/9
Age (years) **Mean (SD)**	69.91 (7.80)
Height (cm) **Mean (SD)**	166.36 (7.62)
Weight (kg) **Mean (SD)**	78.91 (11.93)
BMI (kg/m^2) **Mean (SD)**	28.27 (3.99)
Smokers	
Current Smoker (n)	2
Previous Smoker (n)	6
Never (n)	3
Diabetes	
T1DM (n)	2
T2DM (n)	4
Non-diabetic (n)	5

6.2.2.1 Central haemodynamics

Blood pressure and heart rate were recorded to assess central haemodynamics at baseline and post one INP session (Table 6.2.8). Blood pressure did not significant change after one session of INP however heart rate significantly decreased from a mean of 76bpm at baseline to 71bpm post INP ($p = 0.002$).

Table 6.2.8: ***Recorded central haemodynamics in PAD patients at baseline and post one INP session.*** Vital central haemodynamics measured were heart rate, systolic blood pressure and diastolic blood pressure. Measurements were taken at baseline and after one INP session. Normally distributed data was analysed with paired samples t-test. N = 11. SD = standard deviation, bpm = beats per minute.

	Baseline	**Post one INP session**	**p-value**
Systolic blood pressure (mmHg) Mean (SD)	148.82 (14.38)	142.73 (16.99)	0.200
Diastolic blood pressure (mmHg) Mean (SD)	74.09 (10.20)	73.00 (6.42)	0.754
Heart rate (bpm) Mean (SD)	76.27 (13.85)	70.55 (15.31)	**0.002**

6.2.2.2 Arterial stiffness

Arterial stiffness was assessed at baseline and after one INP session by measuring augmentation index (AIx), augmentation index adjusted for 75bpm (AIx75) and pulse wave velocity (PWV) (Table 6.2.9). A small rise in AIx was observed post INP compared to baseline however this was not significant. When adjusted for heart rate, AIx75 remained consistent between time points. Similarly, measurements obtained at the two time points for PWV were similar.

Table 6.2.9: ***Arterial stiffness measurements obtained from PAD patients at baseline and post one INP session.*** Arterial stiffness was assessed by measuring augmentation index (AIx), AIx adjusted for 75bpm and pulse wave velocity (PWV) at baseline and post one INP session. Paired samples t-test was used to analyse data. Ax and Ax75 n = 11, PWV n = 8. SD = standard deviation, Ax = augmentation index, PWV = pulse wave velocity, m/s = meters per second.

	Baseline	**Post one INP session**	**p-value**
Augmentation index (AIx) Mean (SD)	30.36 (8.38)	33.27 (7.90)	0.324
Augmentation index@75bpm Mean (SD)	30.36 (8.85)	30.27 (13.02)	0.975
Pulse wave velocity (m/s) Mean (SD)	8.88 (2.06)	8.24 (3.08)	0.296

6.2.2.3 Endothelial function

Endothelial function was assessed in the dorsum of the foot of patients at baseline and after one INP session, as previously described (Table 6.2.10). Baseline ACh perfusion remained consistent between the two time points. Peak perfusion in response to ACh increased from a mean of 66.66 at baseline to 88.67 post INP ($p = 0.011$). Similarly, percentage change in perfusion increased from a mean of 124% at baseline to 196% after one INP session ($p = 0.043$) (Figure 6.2.2). No statistically significant changes in baseline SNP perfusion, peak perfusion in response to SNP or SNP percentage change in perfusion were observed between the two time points.

Table 6.2.10: ***Endothelial function of the foot in PAD patients at baseline and post one INP session.*** Cutaneous endothelial function was assessed on the dorsum of the foot of PAD patients using iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP). Normally distributed data was compared using paired samples t-test (a). Non-normally distributed data was compared using Wilcoxon test (b). N = 10. SD = standard deviation, IQR = interquartile range, AU = arbitrary units, ACh = acetylcholine, SNP = sodium nitroprusside.

	Baseline	**Post one INP session**	**p-value**
Baseline ACh Perfusion (au) Mean (SD)	32.49 (13.46)	34.10 (13.86)	0.628[a]
Peak ACh Perfusion (au) Mean (SD)	66.66 (20.20)	88.67 (26.40)	**0.011[a]**
Percentage Change ACh (%) Mean (SD)	124.31 (87.60)	196.19 (136.12)	**0.043[a]**
Baseline SNP Perfusion (au) Median (IQR)	32.70 (14.22)	26.90 (10.90)	0.374[b]
Peak SNP Perfusion (au) Median (IQR)	54.05 (16.20)	69.85 (47.85)	0.202[b]
Percentage Change SNP (%) Median (IQR)	66.23 (103.19)	84.56 (205.66)	0.169[b]

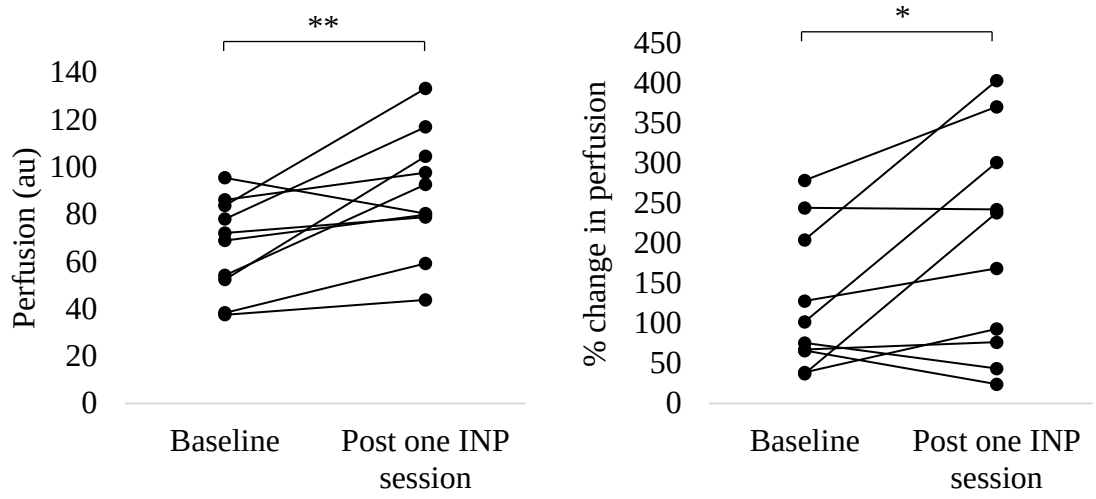

Figure 6.2.2 ***Peak foot blood perfusion and percentage change in foot blood perfusion in response to ACh in PAD patients before and after one session of INP.*** Data were compared using paired samples t-test. ** = $p < 0.01$, * = $p < 0.05$. n = 10. INP = intermittent negative pressure, au = arbitrary units, ACh = acetylcholine.

6.2.2.4 Foot blood perfusion and skin temperature

Cutaneous blood perfusion of the dorsum of the foot in PAD patients was assessed using laser Doppler imaging at baseline and after one INP session (Table 6.2.11) (Figure 6.2.3). There was a significant increase in perfusion from a mean of 51.62 at baseline to 63.70 post-INP ($p = 0.035$). Meanwhile, skin temperature remained consistent between the two time points (Table 6.2.11).

Table 6.2.11: ***Cutaneous blood perfusion and skin temperature in the dorsum of the foot of PAD patients outside the pressure chamber before and after one INP session.*** Cutaneous blood perfusion and skin temperature of the dorsum of the foot was measuring using laser Doppler imaging, outside of the pressure chamber at baseline and after one INP session. Data was compared using paired samples t-test. N = 11. SD = standard deviation, au = arbitrary units.

	Baseline	**Post one INP session**	**p-value**
Perfusion (au) Mean (SD)	51.62 (29.56)	63.70 (32.36)	**0.035**
Skin temperature (°C) Mean (SD)	29.62 (1.72)	29.80 (2.73)	0.736

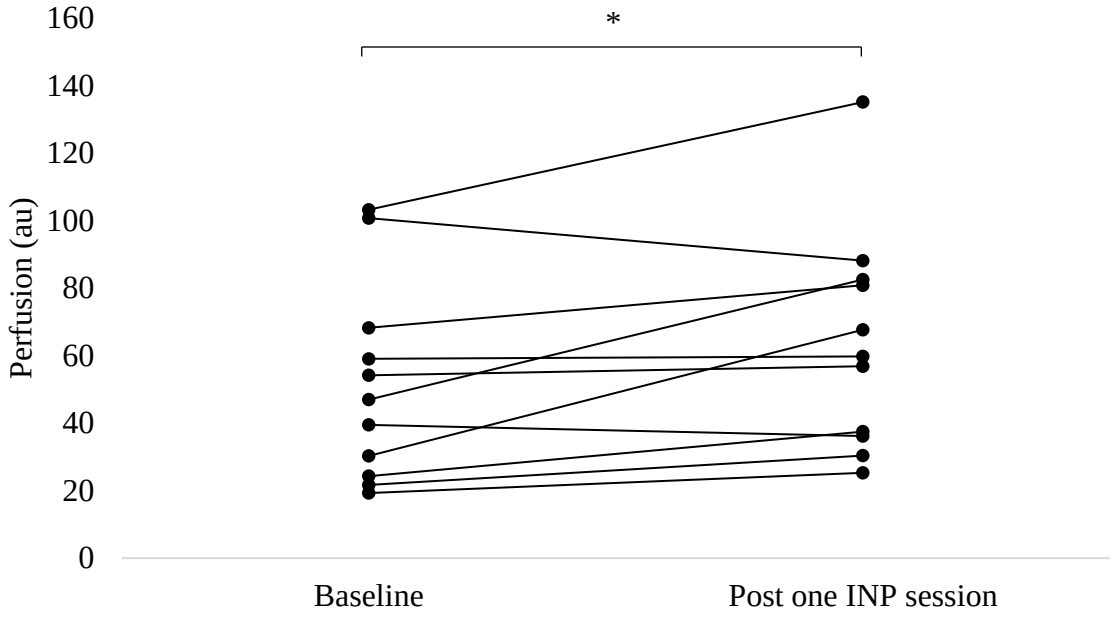

Figure 6.2.3 ***Cutaneous foot blood perfusion in PAD patients before and after one session of INP.*** Data were compared using paired samples t-test. * = $p < 0.05$. n = 11. INP = intermittent negative pressure, au = arbitrary units.

6.2.3 Comparison between baseline and repeated INP

As a larger number of patients were assessed at two time points; baseline and after repeated INP sessions, assessments collected from patients at these two time points were compared against one another. 14 patients were assessed at baseline and post one INP session (Table 6.2.12). 10 of these were male and 4 females. Of these patients, 3 exhibited tissue loss and 11 did not exhibit tissue loss. The mean age of these patients was 72.14 years old. 5 patients had never previously been a smoker while 6 reported being previous smokers and 3 were current smokers. Of all the patients, 0 suffered from type 1 diabetes mellitus, 6 suffered from type 2 diabetes mellitus and 8 did not suffer from diabetes of any kind.

Table 6.2.12: ***Baseline characteristics for patients assessed at baseline and post repeated INP application.*** Baseline characteristics for patients who underwent assessment at two time points: baseline and after repeated INP sessions. SD = standard deviation, BMI = body mass index, T1DM = type 1 diabetes mellitus, T2DM = type 2 diabetes mellitus, PAD = peripheral arterial disease.

N	14
Sex (M/F)	10/4
PAD Status (tissue loss/no tissue loss)	3/11
Age (years) **Mean (SD)**	72.14 (6.89)
Height (cm) **Mean (SD)**	169.00 (8.28)
Weight (kg) **Mean (SD)**	81.50 (21.97)
BMI (kg/m^2) **Mean (SD)**	28.07 (5.96)
Smokers	
Current Smoker (n)	3
Previous Smoker (n)	6
Never (n)	5
Diabetes	
T1DM (n)	0
T2DM (n)	6
Non-diabetic (n)	8

6.2.3.1 Central haemodynamics

Blood pressure and heart rate were recorded at baseline and post repeated INP sessions to assess central haemodynamics (Table 6.2.13). Blood pressure did not significantly change between time points however heart rate significantly decreased from a mean of 69bpm at baseline to 65bpm after repeated INP ($p = 0.021$).

Table 6.2.13: ***Recorded skin temperature and central haemodynamics in PAD patients at baseline and post repeated INP sessions.*** Vital central haemodynamics measured were heart rate, systolic blood pressure and diastolic blood pressure. Measurements were taken at baseline and after repeated INP sessions. Normally distributed data was analysed with paired samples t-test (a). Non-normally distributed data was compared using Wilcoxon test (b). N = 14. SD = standard deviation, IQR = interquartile range, bpm = beats per minute.

	Baseline	**Post repeated INP sessions**	**p-value**
Systolic blood pressure (mmHg) Mean (SD)	145.07 (17.40)	141.36 (22.58)	0.543[a]
Diastolic blood pressure (mmHg) Median (IQR)	71.00 (19.00)	68.00 (19.50)	0.705[b]
Heart rate (bpm) Mean (SD)	68.64 (10.40)	64.64 (9.23)	**0.021[a]**

6.2.3.2 Arterial stiffness

Augmentation index (AIx) remained consistent between the two time points and when adjusted for heart rate, AIx75 also did not significantly change (Table 6.2.14). PWV significantly increased from a baseline mean of 8.17 m/s to 9.23 m/s post repeated INP (p = 0.032) (Figure 6.2.4).

Table 6.2.14: ***Arterial stiffness measurements obtained from PAD patients at baseline and post repeated INP sessions.*** Arterial stiffness was assessed by measuring augmentation index (Ax), Ax adjusted for 75bpm and pulse wave velocity (PWV) at baseline and post repeated INP sessions. Paired samples t-test was used to analyse normally distributed data (a). Wilcoxon test was used to compare non-normally distributed data (b). AIx and AxI75 n = 9, PWV n = 6. SD = standard deviation, AIx = augmentation index, PWV = pulse wave velocity, m/s = meters per second.

	Baseline	**Post repeated INP sessions**	**p-value**
Augmentation index (AIx) Median (IQR)	36.00 (11.50)	37.00 (17.00)	0.812[b]
Augmentation index@75bpm (AIx@75) (SD)	26.11 (10.47)	25.89 (14.15)	0.943[a]
Pulse wave velocity (m/s) Mean (SD)	8.17 (1.78)	9.23 (2.10)	**0.032[a]**

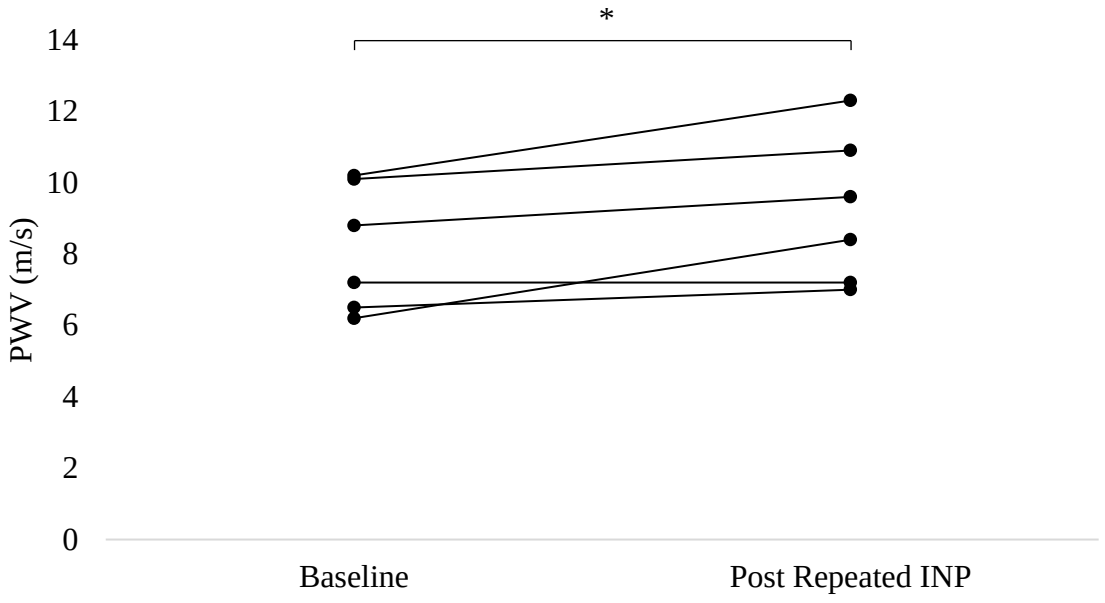

Figure 6.2.4 ***Pulse wave velocity in PAD patients before and after repeated INP application.*** Data were compared using paired samples t-test. * = $p < 0.05$. n = 6. INP = intermittent negative pressure, PWV = pulse wave velocity.

6.2.3.3 Endothelial function

Endothelial function, assessed by iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP), was measured in the dorsum of the foot of patients at baseline and after repeated INP sessions (Table 6.2.15). While an increase in percentage change in perfusion in response to ACh increased to 136% post repeated INP, compared to 109% at baseline, this was not statistically significant. A significant difference in baseline SNP perfusion was recorded between time points ($p = 0.035$). While peak response to SNP did not significantly change after repeated INP, percentage change in perfusion increased from 60% at baseline to 112% post-INP. However, this increase was not statistically significant and may be attributed to the significant increase in baseline SNP perfusion recorded.

Table 6.2.15: ***Endothelial function of the foot in PAD patients at baseline and post repeated INP sessions.*** Cutaneous endothelial function was assessed on the dorsum of the foot of PAD patients using iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP). Normally distributed data was compared using paired samples t-test (a). Non-normally distributed data was compared using Wilcoxon test (b). N = 14. SD = standard deviation, IQR = interquartile range, AU = arbitrary units, ACh = acetylcholine, SNP = sodium nitroprusside.

	Baseline	**Post repeated INP sessions**	**p-value**
Baseline ACh Median (IQR)	34.35 (38.13)	35.85 (25.85)	0.754[b]
Peak ACh Perfusion (au) Median (IQR)	74.45 (38.40)	84.70 (48.48)	0.638[b]
Percentage Change ACh (%) Mean (SD)	109.43 (72.77)	136.48 (93.23)	0.302[a]
Baseline SNP Perfusion (au) Median (IQR)	43.15 (47.78)	30.95 (20.85)	**0.035[b]**
Peak SNP Perfusion (au) Median (IQR)	77.80 (58.28)	74.60 (61.70)	0.470[b]
Percentage Change SNP (%) Median (IQR)	60.26 (141.32)	112.03 (110.64)	0.300[b]

6.2.3.4 Foot blood perfusion and skin temperature

Cutaneous blood perfusion of the dorsum of the foot in PAD patients was assessed using laser Doppler imaging at baseline and after one INP session (Table 6.2.16). Blood perfusion increased after repeated INP sessions (mean of 63.05) compared to baseline (mean of 51.72) however this difference was not statistically significant. Skin temperature of the dorsum of the foot was recorded at baseline and post repeated INP to determine whether any changes in

blood perfusion could be attributed to skin temperature changes however median skin temperature was the same at baseline and after repeated INP application (Table 6.2.16).

Table 6.2.16: ***Cutaneous blood perfusion and skin temperature in the dorsum of the foot of PAD patients outside the pressure chamber before and after repeated INP sessions.*** Cutaneous blood perfusion and skin temperature of the dorsum of the foot was measuring using laser Doppler imaging, outside of the pressure chamber at baseline and after repeated INP sessions. Normally distributed data was compared using paired samples t-test (a). Non-normally distributed data was compared with Wilcoxon singed rank test (b). Perfusion n = 6, skin temperature n = 14. SD = standard deviation, IQR = interquartile range, au = arbitrary units.

	Baseline	**Post repeated INP sessions**	**p-value**
Perfusion (au) Mean (SD)	51.72 (20.85)	63.05 (13.04)	0.306[a]
Skin temperature (°C) Median (IQR)	31.00 (2.40)	31.00 (1.80)	0.887[b]

6.2.4 One-hour INP

In a sub-set of patients (n=11), foot blood perfusion was measured inside the pressure chamber before and after INP application as well as during INP application at three-time points; 10 minutes, 30 minutes, and 60 minutes. This produced five different time points in total.

First, foot blood perfusion measured inside the pressure chamber before and after INP application was analysed (Table 6.2.17). Perfusion increased from a median at baseline of 51.90 to a post-INP median of 62.70 however this difference was not statistically significant.

Table 6.2.17: ***Cutaneous blood perfusion in the dorsum of the foot of PAD patients inside the pressure chamber.*** Cutaneous blood perfusion of the dorsum of the foot was measuring using laser Doppler imaging, inside the pressure chamber at baseline and after one INP session. Data was compared using Wilcoxon test (p = 0.182, n = 11). IQR = interquartile range, au = arbitrary units.

	Baseline	**Post one INP session**	**p-value**
Perfusion (au) Median (IQR)	51.90 (25.40)	62.70 (19.00)	0.182

As described previously in section 6.2.2.4, blood perfusion was also assessed at baseline and after one INP session outside the pressure chamber. A significant increase in perfusion was recorded (Table 6.2.11). To assess whether foot blood perfusion measured outside and increase the pressure chamber were related, correlation analysis was performed. A strong significant positive correlation was found between blood perfusion measured outside at baseline and blood perfusion measured inside at baseline (Pearson correlation, n = 11, r = 0.840, p = 0.001). This was also true for blood perfusion measured outside post INP and blood perfusion inside post INP (Spearman correlation, n = 11, r = 0.873, p = 0.000455). Strong correlations also existed between perfusion measured outside at baseline and inside after one INP session (Spearman correlation, n = 11, r = 0.627, p = 0.039) and between perfusion measured inside at baseline and outside post INP (Pearson correlation, n = 11, r = 0.723, p = 0.012).

As described, blood perfusion was measured inside the pressure chamber at five different time points: before INP application (baseline), at 10 minutes INP, 30 minutes INP, 60 minutes INP and post INP (Table 6.2.18). Median blood perfusion increased at the 10-, 30- and 60-minute time points during INP application compared to baseline. Furthermore, perfusion post INP was greater than baseline with a median of 62.70 and 51.90 respectively. However, no significant differences between the time points were revealed upon statistical analysis with Friedman test (p = 0.055) (Figure 6.2.5).

Table 6.2.18: ***Cutaneous blood perfusion in the dorsum of the foot of PAD patients inside the pressure chamber during INP application.*** Cutaneous blood perfusion of the dorsum of the foot was measured using laser Doppler imaging inside the pressure chamber at five different time points: before INP application, 10 minutes of INP application, 30 minutes INP application, 60 minutes INP application and post INP application. Friedman test was used to compare measurements (p = 0.055, n = 11). IQR = interquartile range, au = arbitrary units.

	Baseline	**10 mins INP**	**30 mins INP**	**60 mins INP**	**Post INP**	**p-value**
Perfusion (au) Median (IQR)	51.90 (25.40)	65.50 (16.40)	67.70 (19.20)	71.50 (27.50)	62.70 (19.00)	0.055

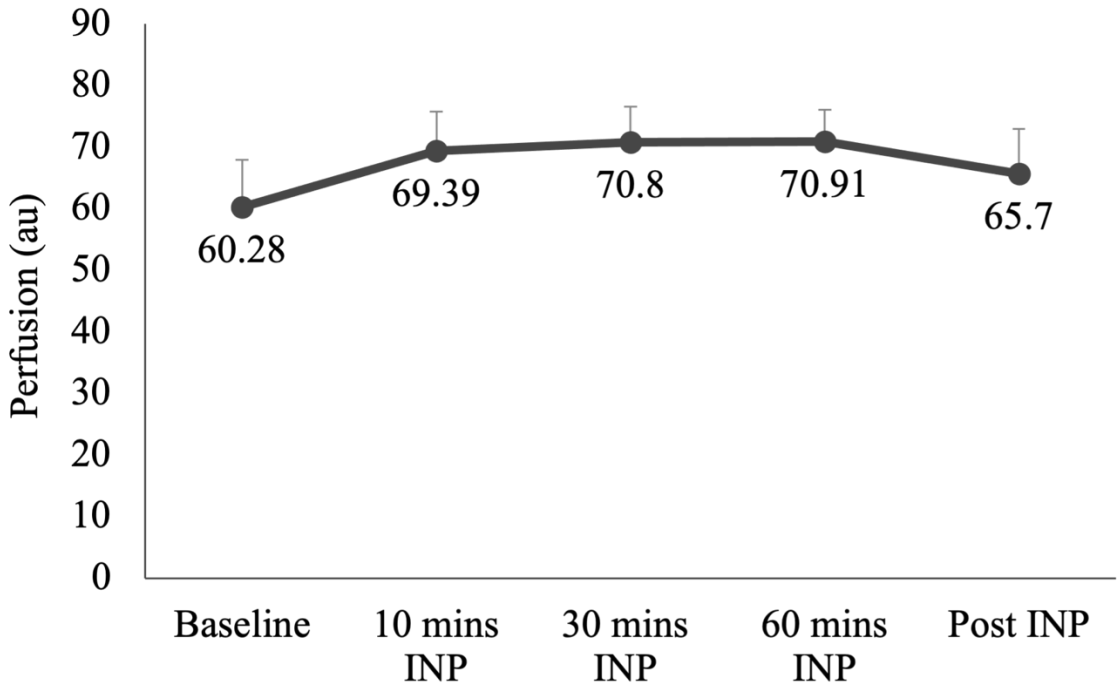

Figure 6.2.5 ***Cutaneous foot blood perfusion in PAD patients before, during and after INP application*** Data is expressed as mean (+/- SEM). Data were compared using Friedman test. n = 11. SEM = standard error of the mean. INP = intermittent negative pressure, au = arbitrary units.

During INP application, fluctuations in blood perfusion were observed in accordance with the negative pressure cycle (Figure 6.2.6).

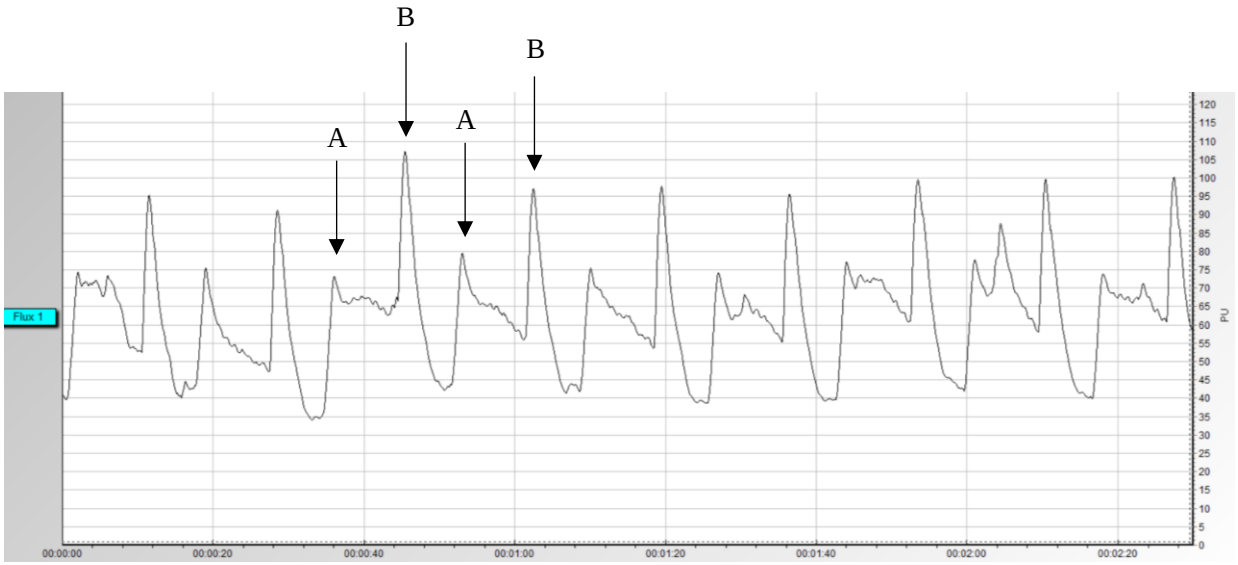

Figure 6.2.6 ***FLPI perfusion trace output during INP application to the foot in PAD patients.*** INP was applied to the foot of patients and blood perfusion measured using FLPI. This trace depicts perfusion (PU) over time as INP is applied. Blood perfusion increased upon application of negative pressure followed by a drop to baseline levels. After 10 seconds of negative pressure application, a rise in perfusion is recorded as pressure is relieved and a fall in perfusion during the 7 seconds of no pressure application. (A) Onset of negative pressure. (B) End of negative pressure and start of atmospheric pressure.

6.2.5 Patients who reported reduced pain

In order to help elucidate factors which may determine if a patient will see a reduction in pain with INP application, analysis was carried out on data collected from patients who reported a reduction in their pain, excluding those patients who reported increased pain or whose pain score remained the same after repeated INP. 13 patients reported a lower pain score after repeated INP versus baseline. Average age of this sub-group of patients was 71.31 with the youngest being 62 and eldest being 78. 10 patients were male and 3 females. 4 patients exhibited tissue loss while 9 did not exhibit any tissue loss. 4 patients were current smokers, 5 previous smokers and 4 patients reported a no smoking history. 1 patient suffered from type 1 diabetes mellitus, 6 reported being diagnosed with type 2 diabetes mellitus and 6 were non-diabetic (Table 6.2.19).

Table 6.2.19: ***Baseline characteristics for a sub-group of patients who reported reduced pain.*** Baseline characteristics for patients who reported a lower pain score after repeated INP compared to baseline. SD = standard deviation, BMI = body mass index, T1DM = type 1 diabetes mellitus, T2DM = type 2 diabetes mellitus.

N	13
Sex (Male/Female)	10/3
PAD Status (tissue loss/no tissue loss)	4/9
Age (years) **Mean (SD)** **(min-max)**	71.31 (4.75) (62-78)
Height (cm) **Mean (SD)**	171.38 (7.78)
Weight (kg) **Mean (SD)**	85.92 (26.86)
BMI (kg/m^2) **Mean (SD)**	28.83 (7.43)
Smokers	
Current Smoker (n)	4
Previous Smoker (n)	5
Never (n)	4
Diabetes	
T1DM (n)	1
T2DM (n)	6
Non-diabetic (n)	6

6.2.5.1 Arterial stiffness

Augmentation index (AIx) and augmentation index adjusted for 75bpm (AIx75) both exhibited the same trend of decreasing after repeated INP application, compared to baseline, however neither of these differences were statistically significant (Table 6.2.20). Pulse wave velocity increased from a baseline mean of 8.50 m/s to a mean of 9.63 m/s post repeated INP however this was not a statistically significant difference (Table 6.2.20).

Table 6.2.20: ***Arterial stiffness measurements obtained from sub-group of PAD patients.*** Arterial stiffness was assessed by measuring augmentation index (AIx), AIx adjusted for 75bpm and pulse wave velocity (PWV) at the two different time points; baseline and after repeated INP sessions. To compare data, paired-samples t-test was used. AIx n = 6, AIx75 n = 6, PWV n = 3. SD = standard deviation, AIx = augmentation index, PWV = pulse wave velocity.

	Baseline	**Post Repeated INP**	**p-value**
Augmentation index (AIx) Mean (SD)	30.67 (12.19)	28.83 (12.73)	0.603
Augmentation index@75bpm Mean (SD)	21.50 (9.93)	19.67 (13.05)	0.684
Pulse wave velocity (m/s) Mean (SD)	8.50 (1.87)	9.63 (2.65)	0.147

6.2.5.2 Pain and ABPI

In patients who reported a lower pain score after repeated INP compared to baseline, mean pain score (rated on a scale of 0-10) was 7.02 at baseline and 4.54 after repeated INP application (p = 0.001) (Table 6.2.21). Ankle-brachial pressure index remained consistent between the two time points with no significant difference being recorded (Table 6.2.21).

Table 6.2.21: ***Pain score and ABPI measurements obtained from a sub-group of PAD patients.*** Pain score (0-10) was reported by patients at baseline and post repeated INP. ABPI was measured in patients baseline and post repeated INP. Wilcoxon test was used to compare data at baseline and post repeated INP. Paired samples t-test was used to analyse data. Pain n = 13, ABPI n = 7. SD = standard deviation, ABPI = ankle-brachial pressure index.

	Baseline	**Post Repeated INP**	**p-value**
Pain score Mean (SD)	7.92 (1.61)	4.54 (2.39)	**0.001**
ABPI Mean (SD)	0.67 (0.31)	0.66 (0.31)	0.688

6.2.5.3 Endothelial function

Endothelial function was assessed in patients who reported a lower pain score after repeated INP application, compared to baseline (Table 6.2.22). No significant differences between the two time points were reported for any of the measurements however some notable differences were recorded. Baseline ACh perfusion, peak ACh perfusion and percentage change in perfusion in response to ACh all remained consistent between time points. Similarly, baseline SNP perfusion and peak SNP perfusion also remained consistent between time points. Percentage change in perfusion in response to SNP was higher after repeated INP compared to baseline (median of 77.65 and 52.15 respectively) however, as stated, this difference was not statistically significant.

Table 6.2.22: ***Endothelial function of the foot in sub-group of PAD patients who reported a lower pain score post-INP compared to baseline.*** Cutaneous endothelial function was assessed on the dorsum of the foot of PAD patients using iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP). Normally distributed data was analysed using paired samples t-test (a) and non-normally distributed data analysed using Wilcoxon test (b). N = 10. SD = standard deviation, IQR = interquartile range, AU = arbitrary units, ACh = acetylcholine, SNP = sodium nitroprusside.

	Baseline	**Post Repeated INP**	**p-value**
Baseline ACh Perfusion (au) Median (IQR)	35.45 (60.80)	37.35 (30.45)	0.683[b]
Peak ACh Perfusion (au) Median (IQR)	75.10 (50.47)	75.95 (40.13)	0.575[b]
Percentage Change ACh (%) Mean (SD)	102.14 (70.33)	115.03 (97.05)	0.709[a]
Baseline SNP Perfusion (au) Median (IQR)	43.15 (72.30)	41.80 (24.40)	0.074[b]
Peak SNP Perfusion (au) Median (IQR)	77.80 (64.75)	79.10 (64.33)	0.508[b]
Percentage Change SNP (%) Median (IQR)	52.15 (77.18)	77.65 (123.70)	0.241[b]

6.2.6 Sub-group analysis

Sub-group analysis of patients was carried out to assess how different groups of patients responded to INP. As number of patients were low, sub-group analysis could only be carried out with pain data.

In a sub-analysis of patients with diabetes and patients without diabetes, pain significantly decreased after repeated INP application, compared to baseline, in patients without but not in patients with diabetes ($p = 0.043$, $= 0.061$ respectively). Comparisons between diabetics and non-diabetics could not be made for other measurements due to low numbers. In a sub analysis of non-smokers and smokers, pain significantly decreased post repeated INP application compared to baseline in both non-smoker and smoker groups ($p = 0.008$, $p = 0.025$ respectively). Between-group comparisons revealed no differences between the two groups at either of the two time points. In a sub-analysis of males and females, pain significantly decreased post repeated INP compared to baseline in males but not females ($p = 0.005$, $p = 0.178$ respectively). While pain score reported by females after repeated INP application was higher in females (mean of 7.80) than males (mean of 5.69), between-group comparisons revealed no significant differences between the two groups at either of the two time points.

6.2.7 Wavelet Analysis

Wavelet analysis was carried out on the FLPI perfusion traces collected from patient's feet at baseline, during INP, after one INP session and after repeated INP sessions. Percentage contribution of each of the six components (cardiac, respiratory, myogenic, sympathetic, endothelial, and non-endothelial) was recorded.

6.2.7.1 Comparison between baseline, one INP session and repeated INP

When comparing the three time points: baseline, after one INP session and after repeated INP, no significant changes in percentage contribution of any of the six components was recorded (Table 6.2.23). The highest contributing component at all three time points was the endothelial component (mean of 33.82%, 31.39% and 29.48% at each respective time point).

Table 6.2.23. ***Percentage contribution of each component in patients at baseline, after one INP session and after repeated INP application.*** Wavelet analysis was carried out on FLPI traces collected from patients at baseline, after one INP session and after repeated INP application. Percentage contribution of each component to the overall signal was recorded and presented. Normally distributed data was compared with paired-samples t-test (a). Non-normally distributed data was analysed with Wilcoxon signed rank test (b). N = 5. IQR = interquartile range, SD = standard deviation.

	Baseline	**Post one INP session**	**Post repeated INP**	**p value**
Cardiac (%) Mean (SD)	0.77 (0.37)	0.67 (0.60)	1.12 (0.74)	0.328
Respiratory (%) Mean (SD)	5.55 (2.60)	5.52 (3.75)	6.98 (4.25)	0.554
Myogenic (%) Mean (SD)	13.96 (3.38)	15.28 (5.89)	15.81 (5.98)	0.385
Sympathetic (%) Median (IQR)	21.09 (6.11)	26.09 (11.20)	23.09 (7.74)	0.074
Endothelial (%) Mean (SD)	33.82 (8.02)	31.39 (9.52)	29.48 (5.56)	0.679
Non-endothelial (%) Mean (SD)	23.10 (9.78)	18.55 (7.58)	22.43 (11.18)	0.686

6.2.7.2 Comparison between baseline and one INP session

When comparing only two time points: baseline and post one INP session, no significant changes in percentage contribution of any of the six components was recorded (Table 6.2.24). The highest contributing component at both time points was the endothelial component (mean of 29.79% at baseline and mean of 30.30% post one INP session).

Table 6.2.24. ***Percentage contribution of each component in patients at baseline and after one INP session.*** Wavelet analysis was carried out on FLPI traces collected from patients at baseline and after one INP session. Percentage contribution of each component to the overall signal was recorded and presented. Normally distributed data was compared with paired-samples t-test (a). Non-normally distributed data was analysed with Wilcoxon signed rank test (b). N = 11. IQR = interquartile range, SD = standard deviation.

	Baseline	**Post one INP session**	**p value**
Cardiac (%) Mean (SD)	1.12 (0.80)	0.74 (0.49)	0.095[a]
Respiratory (%) Mean (SD)	7.12 (4.33)	5.65 (2.83)	0.224[a]
Myogenic (%) Mean (SD)	15.35 (3.67)	16.89 (5.95)	0.484[a]
Sympathetic (%) Median (IQR)	22.32 (10.74)	26.35 (9.59)	0.110[b]
Endothelial (%) Mean (SD)	29.79 (7.69)	30.30 (7.92)	0.886[a]
Non-endothelial (%) Mean (SD)	21.11 (8.98)	17.18 (7.69)	0.268[a]

6.2.7.3 Comparison between baseline and repeated INP

When comparing only two time points: baseline and post repeated INP session, only one significant change was recorded (Table 6.2.25). Percentage contribution of the respiratory component significantly increased form a baseline mean of 9.15% to 11.81% post repeated INP (p=0.034). The highest contributing component at both time points was the myogenic component (mean of 29.31% at baseline, mean of 28.57% after repeated INP).

Table 6.2.25. ***Percentage contribution of each component in patients at baseline and after repeated INP application.*** Wavelet analysis was carried out on FLPI traces collected from patients at baseline and after repeated INP application. Percentage contribution of each component to the overall signal was recorded and presented. Normally distributed data was compared with paired-samples t-test (a). Non-normally distributed data was analysed with Wilcoxon signed rank test (b). N = 14. IQR = interquartile range, SD = standard deviation.

	Baseline	**Post repeated INP**	**p value**
Cardiac (%) Median (IQR)	1.24 (1.04)	1.81 (1.76)	0.177
Respiratory (%) Mean (SD)	9.15 (4.25)	11.81 (7.23)	**0.034**
Myogenic (%) Median (IQR)	16.82 (12.39)	20.39 (13.03)	0.363
Sympathetic (%) Mean (SD)	29.31 (7.59)	28.57 (7.30)	0.794
Endothelial (%) Mean (SD)	27.79 (10.61)	24.12 (8.63)	0.123
Non-endothelial (%) Median (IQR)	9.78 (13.14)	6.23 (19.17)	0.300

6.2.7.4 During INP application

During INP application, the highest contributing component in patients was the myogenic component across all three time points (Table 6.2.26). No statistically significant changes in percentage contribution of any of the six components was recorded across the three time points.

Table 6.2.26: ***Percentage contribution of each component in patients during INP application.*** Wavelet analysis was carried out on the FLPI perfusion traces collected from patients during INP application. Percentage contribution of each component to the overall signal was recorded. Normally distributed data was compared using repeated measures ANOVA (a) and non-normally distributed data analysed with Friedman test (b). N = 10. IQR = interquartile range, SD = standard deviation.

	10 mins INP	**30 mins INP**	**60 mins INP**	**p value**
Cardiac (%) Median (IQR)	0.98 (0.91)	1.00 (0.41)	1.00 (0.90)	0.905[b]
Respiratory (%) Mean (SD)	12.76 (5.53)	13.48 (5.98)	13.15 (5.78)	0.941[a]
Myogenic (%) Mean (SD)	31.06 (13.46)	28.90 (8.63)	27.85 (9.54)	0.601[a]
Sympathetic (%) Mean (SD)	18.96 (5.30)	19.18 (5.31)	18.92 (4.96)	0.990[a]
Endothelial (%) Mean (SD)	21.94 (8.98)	22.57 (7.20)	24.01 (7.76)	0.672[a]
Non-endothelial (%) Mean (SD)	14.32 (6.93)	14.87 (4.65)	14.89 (5.98)	0.963[a]

6.2.8 Blood inflammatory and oxidative stress markers

Blood samples collected from PAD patients were tested for blood biomarkers of inflammation and oxidative stress namely ICAM-1, MMP-8, VCAM-1, IL-1a, E-selectin and MPO in order to evaluate the systemic effects of repeated INP application (Table 6.2.27). In PAD patients, levels of all measured biomarkers did not significantly change after repeated INP application, compared to baseline.

Table 6.2.27 ***Blood borne inflammatory and oxidative stress biomarkers at baseline and after repeated INP in PAD patients.*** Inflammatory and oxidative stress markers ICAM-1, MMP-8, VCAM-1, IL-1a, E-selectin and MPO were measured in serum and plasma samples obtained from PAD patients at baseline and after repeated INP application for up to 8 weeks. Normally distributed data was compared using paired-samples t-test. n = 9. ICAM-1 = intercellular adhesion molecule 1, MMP-8 = matrix metalloproteinase 8, VCAM-1 = vascular cell adhesion protein 1, IL-1a = interleukin-1 alpha, E-selectin = endothelial-selectin, MPO = myeloperoxidase, SD = standard deviation.

	Baseline	**Post repeated INP**	**p-value**
ICAM-1 (ug/ml) Mean (SD)	1.65 (0.50)	1.56 (0.50)	0.480
MMP-8 (ug/ml) Mean (SD)	$9.8x10^{-3}$ ($4.1x10^{-3}$)	$9.6x10^{-3}$ ($3.0x10^{-3}$)	0.842
VCAM-1 (ug/ml) Mean (SD)	1.85 (0.37)	1.82 (0.37)	0.750
IL-1a (ug/ml) Mean (SD)	$2.3x10^{-4}$ ($5.9x10^{-5}$)	$2.2x10^{-4}$ ($6.0x10^{-5}$)	0.761
E-selectin (ug/ml) Mean (SD)	0.08 (0.02)	0.08 (0.02)	0.989
MPO (ug/ml) Mean (SD)	0.53 (0.022)	0.60 (0.50)	0.668

6.2.9 Summary of findings

- 21 PAD patients, 4 with tissue loss and 16 without tissue loss, were recruited and assessed within the study.
- Five patients completed assessment at all three time points; baseline, after one INP session and after repeated INP. 11 patients were assessed at two of the time points; baseline and after one INP session while 14 patients were assessed at baseline and after repeated INP application.
- Pain significantly decreased after repeated INP sessions.
- Upon sub-group analysis, pain significantly decreased in those without diabetes but not those with diabetes and in men but not in women.

- No changes in ABPI were recorded.
- Microvascular blood perfusion and endothelial function significantly improved after one INP session.
- Microvascular blood perfusion and endothelial function improved after repeated INP sessions however not significantly.
- PWV significantly increased after repeated INP versus baseline.
- No significant changes in contribution of any of the six vasomotion components was recorded between time points.
- No changes in levels of blood borne inflammatory or oxidative stress markers was recorded after repeated INP application, versus baseline.

6.3 Cold Hands Study

50 volunteers were recruited in total to the cold hands study. 25 of these were healthy volunteers which served as controls and 25 reported experiencing excessively cold hands and Raynaud's-like symptoms, but not formally diagnosed as suffering from Raynaud's phenomenon. These two groups will herein by named 'control' and 'cold hands'. The groups were sex matched with 9 males and 16 females in each group (Table 6.3.1). Each group contained 2 current cigarette smokers. The median age of healthy volunteers was 23 years old and median age of cold hand volunteers was 21 years old. When comparing the two groups, significant differences were present for age, weight and body mass index (BMI). Weight and BMI were both significantly lower in the cold hands group compared to the healthy group (p=0.002 and p=0.001, respectively).

Table 6.3.1: ***Baseline characteristics for recruited participants and between group differences.*** Comparison for between groups using independent samples t-test for normally distributed data (a). Non-normally distributed data was compared using Mann-Whitney U Test (b). IQR = interquartile range, SD = standard deviation, BMI = body mass index.

	Control	**Cold hands**	**p-value**
Total number n	25	25	
Age (years) Median (IQR) (min-max)	23 (5.00) (18-31)	21 (4.00) (18-29)	**0.012[b]**
Sex			
Male	9/25	9/25	
Female	16/25	16/25	
Height (cm) Mean (SD)	169.68 (8.20)	168.00 (12.51)	0.577[a]
Weight (kg) Mean (SD)	71.08 (12.78)	60.50 (9.53)	**0.002[a]**
BMI (kg/m^2) Mean (SD)	24.57 (3.47)	21.46 (3.02)	**0.001[a]**
Number of current smokers	2	2	

6.3.1 Comparison between Controls and Cold Hand Volunteers

Between group comparisons were made for all measurements at both time points; before and after INP application (Table 6.3.2). No significant between-group differences were found for skin temperature, blood pressure, heart rate, AIx, AIx75 or PWV at either of the time points however notable differences were observed. AIx was higher in the control group than the cold hand group at baseline (median of 8.00 and 3.00 respectively). The true was same for AIx75 with the control group median being 4.50 and cold hand group median being 1.00. However, the opposite was true for measurements obtained post-INP with both AIx and AIx75 being higher in the cold hands group than the control group. Although not significant, PWV was higher in the control group before and after INP application compared to the cold hands group. No significant differences between control and cold hand groups were observed for endothelial function measurements for either of the two time points. Forearm blood perfusion was also consistent between groups before and after INP application.

Table 6.3.2: ***Comparison between control and cold hand volunteer groups for all measurements.*** Measurements obtained in each group obtained at each of the two time points were compared against each other to assess any between-group differences. Normally distributed data was compared using independent samples t-test. Non-normally distributed data was compared using Mann-Whitney U test. N = 25 per group. Within group analysis comparing time points was conducted (* = $p < 0.05$, ** = $p < 0.01$, *** $p = <0.001$). SD = standard deviation, IQR = interquartile range, C = Celsius, bpm = beats per minute, AIx = augmentation index, AIx@75 = augmentation index @ 75bpm, PWV = pulse wave velocity, au = arbitrary units, ACh = acetylcholine, SNP = sodium nitroprusside.

		Control	Cold	P Value
Skin Temperature (ºC)	**Before Mean (SD)**	32.66 (1.38)	32.64 (1.64)	0.963
	After Median (IQR)	32.80 (2.50)	33.00 (2.20)	0.786
Systolic Blood Pressure (mmHg)	**Before Mean (SD)**	115.88 (9.52)	118.16 (13.51)	0.494
	After Mean (SD)	117.08 (11.42)	114.60 (9.70)	0.412
Diastolic Blood Pressure (mmHg)	**Before Mean (SD)**	67.24 (6.88)	67.36 (10.76)	0.963
	After Mean (SD)	68.72 (8.30)	66.52 (6.92)	0.314
Heart Rate (bpm)	**Before Median (IQR)**	62.00 (10.50)	59.00 (16.50)	0.756
	After Mean (SD)	61.20 (10.47)	61.24 (9.34)	0.989
Augmentation Index (%AIx)	**Before Median (IQR)**	8.00 (16.50)	3.00 (16.00)	0.285
	After Mean (SD)	5.96 (12.85)	6.76 (9.94)	0.807
Ax@75 (%AIx75)	**Before Median (IQR)**	4.50 (14.75)	1.00 (17.50)	0.368
	After Mean (SD)	-0.54 (14.43)	0.04 (12.65)	0.881

Pulse Wave Velocity (PWV) (ms^{-1})	**Before Median (IQR)**	5.45 (1.25)	4.60 (1.30)	0.087
	After Mean (SD)	5.35 (1.07)	4.93 (1.15)	0.194
ACh Baseline Perfusion (au)	**Before Median (IQR)**	41.20 (13.45)	38.50 (17.50)	0.938
	After Median (IQR)	54.40 (28.40)**	49.10 (24.60)**	0.461
ACh Peak Perfusion (au)	**Before Mean (SD)**	142.92 (39.27)	144.30 (36.69)	0.898
	After Median (IQR)	151.80 (90.50)	151.40 (61.85)	0.839
Percentage change ACh (%)	**Before Mean (SD)**	245.31 (92.35)	252.59 (97.61)	0.788
	After Mean (SD)	185.86 (87.56)*	202.25 (104.97)*	0.552
SNP Baseline Perfusion (au)	**Before Mean (SD)**	49.70 (11.91)	43.89 (14.43)	0.127
	After Median (IQR)	52.00 (28.35)*	49.40 (21.55)*	0.277
SNP Peak Perfusion (au)	**Before Mean (SD)**	150.20 (38.44)	149.37 (50.56)	0.948
	After Mean (SD)	140.98 (44.30)	137.50 (43.96)	0.781
Percentage change SNP (%)	**Before Mean (SD)**	217.32 (104.36)	254.94 (99.30)	0.198
	After Mean (SD)	148.37 (70.29)**	172.84 (73.68)***	0.235
Forearm Perfusion (au)	**Before Median (IQR)**	77.00 (19.0)	75.40 (36.3)	0.545
	After Mean (SD)	81.02 (20.63)	71.99 (21.68)	0.353

6.3.2 One-hour INP

Both groups used the INP device for one hour. During this time, cutaneous blood perfusion of the hand and fingertips were measured using laser Doppler at three different time points; at 10 minutes INP application, 30 minutes INP application and 60 minutes INP application (Table 6.3.3). In the control group, blood perfusion of the hand remained consistent between all three time points. In all fingers, blood perfusion was lower at 30 minutes compared to 10 minutes. In fingers 1, 2 and 3, perfusion was lower once again at 60 minutes compared to 30 minutes. However, no significant differences between the time points for any hand or finger perfusion recording was recorded. In cold hand volunteers, blood perfusion of the hand remained consistent between all three time points. Similar to the trend seen in control volunteers, In all fingers, blood perfusion was lower at 30 minutes compared to 10 minutes. However, no significant differences between the time points for any hand or finger perfusion recording was recorded. When comparing between groups, no significant differences for any of time points in either the hand or fingers was detected.

Table 6.3.3 ***Comparison of cutaneous perfusion between control and cold hand volunteer groups during INP application.*** Measurements obtained in each group obtained at each time points during INP application were compared against each other to assess any between-group differences. Normally distributed data was compared using independent samples t-test. Non-normally distributed data was compared using Mann-Whitney U test. N = 20. SD = standard deviation, IQR = interquartile range, au = arbitrary units.

			Control	**Cold Hands**	**p-value**
Perfusion (au)	**Hand**	**10 mins Median (IQR)**	105.45 (57.3)	96.65 (95.0)	0.705
		30 mins Median (IQR)	123.10 (59.1)	99.80 (79.7)	0.762
		60 mins Median (IQR)	113.10 (69.6)	96.80 (82.4)	0.821
	Finger 1	**10 mins Median (IQR)**	188.00 (134.0)	155.10 (115.2)	0.226
		30 mins Median (IQR)	165.35 (152.3)	150.50 (110.2)	0.496
		60 mins Median (IQR)	175.0 (132.6)	156.95 (117.6)	0.545
	Finger 2	**10 mins Median (IQR)**	188.45 (176.90)	150.05 (148.4)	0.821
		30 mins Median (IQR)	178.15 (192.8)	146.35 (113.7)	0.762

		60 mins Median (IQR)	146.25 (86.3)	147.25 (93.40)	0.880
	Finger 3	**10 mins Median (IQR)**	211.25 (211.5)	134.45 (128.0)	0.762
		30 mins Median (IQR)	170.0 (172.7)	131.30 (101.80)	0.496
		60 mins Median (IQR)	156.0 (143.7)	134.90 (75.5)	1.00
	Finger 4	**10 mins Mean (SD)**	216.89 (109.98)	184.13 (97.92)	0.491
		30 mins Median (IQR)	153.90 (165.9)	149.60 (98.7)	0.496
		60 mins Median (IQR)	154.75 (158.9)	132.40 (101.80)	0.940

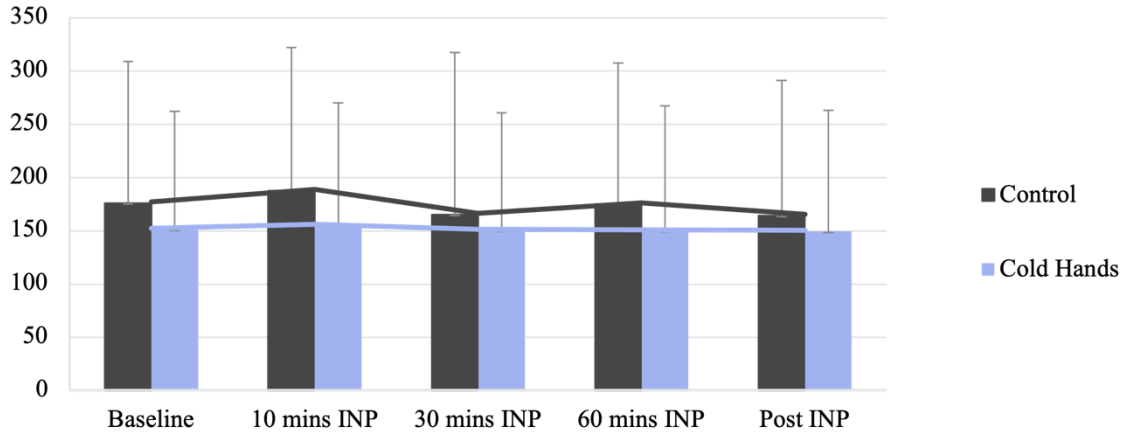

Figure 6.3.1 ***Cutaneous blood perfusion in cold hands and control participants before, during and after INP application*** Data is expressed as mean +/- SEM. Data were compared using Friedman test. n = 11. SEM = standard error of the mean. INP = intermittent negative pressure, au = arbitrary units.

6.3.3 Male and Female Groups

As Raynaud's Phenomenon is significantly more prevalent in females, analysis was conducted in sub-groups of males and females. In total, 18 males and 32 females were recruited to the study.

6.3.3.1 Comparison between control males and cold hand males

Between group comparisons were made between control males and cold hand males (Table 6.3.4). 9 males were recruited to each group and compared. Significant differences in weight and body mass index (BMI) were recorded with both weight and BMI both being lower in cold hand males than control males.

Table 6.3.4 ***Baseline characteristics for recruited control males and cold hand males.*** Comparison for between groups was conducted using independent samples t-test for normally distributed data. SD = standard deviation, BMI = body mass index.

	Control Males	**Cold Males**	**p-value**
Total number n	9	9	
Age (years) Mean (SD)	25.56 (3.36)	22.67 (4.06)	0.120
Height (cm) Mean (SD)	177.00 (6.75)	178.89 (4.76)	0.502
Weight (kg) Mean (SD)	82.22 (9.69)	67.11 (9.09)	**0.004**
BMI (kg/m^2) Mean (SD)	26.20 (2.77)	20.96 (3.14)	**0.002**
Number of current smokers	1	2	

Comparisons between control and cold hand males were made for each measurement at each of the two time points (Table 6.3.5). No statistically significant differences were observed between the two groups at either of the time points however some notable differences were observed. AIx was higher in control males than cold hand males at baseline however after INP application, AIx was higher in the cold hand group than control. The same trend was seen for AIx75 although none of these differences were significant. No notable differences were recorded between control and cold hand males at either of the two time points.

Table 6.3.5: ***Comparison between control male and cold male groups for all measurements.*** Measurements obtained from control and cold males before and after INP application were compared using independent samples t-test for normally distributed data (a) and Mann-Whitney for non-normally distributed data (b). Within group analysis comparing time points was conducted (* = $p < 0.05$, ** = $p < 0.01$). SD = standard deviation, IQR = interquartile range, C = Celsius, bpm = beats per minute, AIx = augmentation index, PWV = pulse wave velocity, au = arbitrary units, ACh = acetylcholine, SNP = sodium nitroprusside.

		Control Males	**Cold Males**	**P-value**
Systolic blood pressure (mmHg)	**Before Median (IQR)**	121.00 (13.00)	128.00 (11.00)	0.170[b]
	After Mean (SD)	120.89 (13.32)	123.78 (6.20)	0.567[a]
Diastolic blood pressure (mmHg)	**Before Median (IQR)**	66.00 (12.50)	68.00 (14.00)	0.757[b]
	After Mean (SD)	66.89 (8.28)	68.33 (5.57)	0.670[a]
Heart Rate (bpm)	**Before Median (IQR)**	59.00 (14.50)	59.00 (25.50)	0.863[b]
	After Mean (SD)	56.33 (10.39)	60.33 (9.50)	0.407[a]
Skin temperature (°C)	**Before Mean (SD)**	32.62 (1.65)	33.30 (1.10)	0.321[a]
	After Mean (SD)	32.69 (2.04)	33.67 (1.89)	0.307[a]
Augmentation index (%AIx)	**Before Median (IQR)**	10.00 (18.50)	3.00 (12.50)	0.258[b]
	After Mean (SD)	6.78 (11.85)	7.33 (8.38)	0.910[a]
Augmentation Index @ 75bpm (%AIx75)	**Before Mean (SD)**	1.67 (10.14)	-0.78 (9.76)	0.609[a]
	After Mean (SD)	-2.22 (12.82)	-.56 (11.79)	0.639[a]
	Before	6.23 (0.91)	6.34 (1.63)	0.857[a]

Pulse wave velocity (m/s)	**Mean (SD)**			
	After Median (IQR)	5.70 (1.72)	5.80 (1.95)	0.531[b]
ACh Baseline Perfusion (au)	**Before Mean (SD)**	44.98 (15.19)	43.83 (13.07)	0.866[a]
	After Median (IQR)	49.70 (40.90)	63.10 (21.65)**	0.627[b]
ACh Peak Perfusion (au)	**Before Mean (SD)**	142.76 (30.69)	133.48 (34.27)	0.554[a]
	After Median (IQR)	139.40 (45.65)	161.00 (51.25)**	0.309[b]
Percentage change ACh (%)	**Before Mean (SD)**	234.97 (77.89)	230.23 (138.31)	0.930[a]
	After Mean (SD)	181.19 (94.23)	186.73 (140.21)*	0.923[a]
SNP Baseline Perfusion (au)	**Before Mean (SD)**	48.37 (10.43)	47.27 (12.95)	0.845[a]
	After Mean (SD)	61.71 (23.83)	56.89 (28.16)	0.700[a]
SNP Peak Perfusion (au)	**Before Mean (SD)**	158.16 (47.92)	144.26 (36.18)	0.497[a]
	After Mean (SD)	138.16 (51.24)	134.76 (33.90)	0.870[a]
Percentage change SNP (%)	**Before Mean (SD)**	237.77 (117.19)	226.73 (112.47)	0.841[a]
	After Mean (SD)	135.60 (74.08)	162.05 (63.07)*	0.427[a]
Forearm Perfusion (au)	**Before Mean (SD)**	83.47 (22.17)	73.05 (22.39)	0.437[a]
	After Mean (SD)	82.52 (24.93)	75.47 (22.34)	0.617

6.3.3.2 Comparison between control females and cold hand females

Between group comparisons were made between control females and cold hand females (Table 6.3.6). 16 females were recruited to each group and compared. Significant differences in age and weight were recorded between groups. Cold hand females were significantly lighter in weight than control females (56.78kg and 64.81kg respectively) (p = 0.015).

Table 6.3.6: ***Baseline characteristics for control females and cold hand females.*** Comparison for between groups was conducted using independent samples t-test for normally distributed data (a). Non-normally distributed data was compared using Mann-Whitney U Test (b). IQR = interquartile range, SD = standard deviation, BMI = body mass index.

	Control Females	**Cold Females**	**p-value**
Total number n	16	16	
Age (years) Median (IQR)	22.00 (2.00)	20.50 (3.00)	**0.043[b]**
Height (cm) Mean (SD)	165.56 (5.75)	161.88 (11.28)	0.253[a]
Weight (kg) Mean (SD)	64.81 (9.75)	56.78 (7.76)	**0.015[a]**
BMI (kg/m^2) Median (IQR)	22.60 (3.90)	21.40 (3.80)	0.073[b]
Number of current smokers	1	0	

Comparisons between control and cold hand females were made for each measurement at each of the two time points (Table 6.3.7). No significant differences were recorded between the two female groups at either of the two time points. Although forearm perfusion was lower in the cold hand females both at baseline and after INP application compared to healthy females, these differences were not significantly different.

Table 6.3.7: ***Comparison between control female and cold hand female volunteer groups for all measurements.*** Measurements obtained from control and cold hand females before and after INP application were compared using independent samples t-test for normally distributed data (a) and Mann-Whitney for non-normally distributed data (b). Within group analysis comparing time points was conducted (* = $p < 0.05$, ** = $p < 0.01$).SD = standard deviation, IQR = interquartile range, C = Celsius, bpm = beats per minute, AIx = augmentation index, PWV = pulse wave velocity, au = arbitrary units, ACh = acetylcholine, SNP = sodium nitroprusside.

		Control Females	**Cold Females**	**P-value**
Systolic blood pressure (mmHg)	**Before Mean (SD)**	113.75 (9.77)	112.56 (9.97)	0.736[a]
	After Mean (SD)	114.94 (10.02)	109.44 (7.15)	0.084[a]
Diastolic blood pressure (mmHg)	**Before Mean (SD)**	67.56 (6.96)	65.69 (10.04)	0.544[a]
	After Mean (SD)	69.75 (8.40)	65.50 (8.40)	0.143[a]
Heart Rate (bpm)	**Before Median (IQR)**	63.00 (8.00)	59.50 (16.50)	0.539[b]
	After Mean (SD)	63.94 (9.77)	61.75 (9.53)	0.526[a]
Skin temperature (°C)	**Before Mean (SD)**	32.68 (1.26)	32.26 (1.80)	0.459[a]
	After Mean (SD)	32.35 (1.56)	32.06 (2.16)	0.662[a]
Augmentation index (%AIx)	**Before Mean (SD)**	7.81 (8.84)	8.31 (13.39)	0.902[a]
	After Mean (SD)	5.50 (13.73)	6.44 (10.97)	0.832[a]
Augmentation Index @ 75bpm (%AIx75)	**Before Mean (SD)**	3.67 (8.89)	3.13 (15.30)	0.906[a]
	After Mean (SD)	0.47 (15.65)	-0.25 (13.48)	0.892[a]

Pulse wave velocity (m/s)	**Before Mean (SD)**	4.96 (1.02)	4.39 (0.59)	0.062[a]
	After Mean (SD)	4.95 (0.91)	4.37 (0.68)	0.050[a]
ACh Baseline Perfusion (au)	**Before Median (IQR)**	41.50 (10.32)	37.85 (14.90)	0.985[b]
	After Median (IQR)	58.80 (25.78)**	46.20 (21.53)	0.109[b]
ACh Peak Perfusion (au)	**Before Mean (SD)**	143.00 (44.32)	150.38 (37.66)	0.616[a]
	After Median (IQR)	156.60 (101.85)	150.75 (41.28)	0.386[b]
Percentage change ACh (%)	**Before Mean (SD)**	251.12 (101.54)	265.17 (67.64)	0.649[a]
	After Mean (SD)	188.48 (86.67)	210.98 (83.19)	0.406[a]
SNP Baseline Perfusion (au)	**Before Mean (SD)**	50.46 (12.94)	41.99 (15.26)	0.101[a]
	After Mean (SD)	57.01 (16.67)	52.88 (24.22)*	0.579[a]
SNP Peak Perfusion (au)	**Before Mean (SD)**	145.73 (32.86)	152.25 (58.04)	0.689[a]
	After Mean (SD)	142.57 (41.62)	139.04 (49.72)	0.829[a]
Percentage change SNP (%)	**Before Mean (SD)**	205.82 (98.54)	270.80 (91.01)	0.062[a]
	After Median (IQR)	152.47 (48.16)	163.21 (94.48)**	0.187[b]
Forearm Perfusion (au)	**Before Mean (SD)**	80.60 (9.24)	72.25 (17.29)	0.427[a]
	After Mean (SD)	78.78 (15.17)	66.78 (22.74)	0.414[a]

6.3.4 Wavelet Analysis

6.3.4.1 Controls and Cold Hand Volunteers

Wavelet analysis was carried out on the FLPI perfusion traces collected from participants at baseline, during INP and after one INP session. Percentage contribution of each component was recorded. When comparing baseline to after one INP session in controls and cold hand volunteers, no significant differences in percentage contribution of any of the six components was recorded. In both groups at both time points, the greatest contribution was from the endothelial component. When comparing between groups, no significant differences for any of the components at either time point was recorded (Table 6.3.8). During INP application, the highest contributing component in both groups was the myogenic component across all three time points. No significant changes in contribution of each component were recorded between the three time points in controls and cold hand volunteers. No significant between group differences were recorded for any of the three time points (Table 6.3.9).

Table 6.3.8: ***Between group comparisons for each vasomotion component before and after INP application.*** Measurements obtained from controls and cold hand volunteers at baseline and after one INP session were compared. Normally distributed data was compared using independent samples t-test (a). Non-normally distributed data was compared using Mann-Whitney U test (b). IQR = interquartile range, SD = standard deviation.

		Control	Cold	p value
Cardiac (%)	**Before Median (IQR)**	1.31 (2.02)	1.23 (0.72)	0.364[b]
	After Median (IQR)	1.33 (0.98)	1.27 (1.01)	0.762[b]
Respiratory (%)	**Before Mean (SD)**	5.72 (2.30)	5.76 (1.68)	0.969[a]
	After Mean (SD)	5.44 (2.22)	5.05 (1.74)	0.664[a]
Myogenic (%)	**Before Mean (SD)**	16.17 (4.98)	16.37 (5.69)	0.934[a]
	After Median (IQR)	18.06 (7.56)	14.14 (4.56)	0.450[b]
Sympathetic (%)	**Before Median (IQR)**	24.18 (7.80)	23.51 (6.51)	0.545[b]
	After Mean (SD)	22.12 (4.04)	24.17 (2.78)	0.085[a]
Endothelial (%)	**Before Mean (SD)**	31.87 (6.23)	32.81 (7.07)	0.755[a]
	After Mean (SD)	29.70 (3.31)	31.81 (5.35)	0.304[a]
Non-endothelial (%)	**Before Median (IQR)**	22.62 (7.84)	18.59 (6.29)	0.199[b]
	After Mean (SD)	23.39 (7.07)	21.70 (4.59)	0.534[a]

Table 6.3.9: ***Between group comparisons for each vasomotion component during INP application.*** Measurements obtained from controls and cold hand volunteers during INP application were compared. Normally distributed data was compared using independent samples t-test (a). Non-normally distributed data was compared using Mann-Whitney U test (b). IQR = interquartile range, SD = standard deviation.

		Control	Cold	p value
Cardiac (%)	**10 mins Mean (SD)**	1.28 (0.35)	1.08 (0.31)	0.187[a]
	30 mins Mean (SD)	1.37 (0.40)	1.18 (0.20)	0.191[a]
	60 mins Median (IQR)	1.39 (0.52)	1.25 (0.26)	0.677[b]
Respiratory (%)	**10 mins Mean (SD)**	21.58 (7.83)	19.24 (6.81)	0.485[a]
	30 mins Mean (SD)	22.19 (7.29)	20.11 (5.43)	0.479[a]
	60 mins Mean (SD)	22.70 (9.18)	21.69 (5.88)	0.771[a]
Myogenic (%)	**10 mins Mean (SD)**	35.82 (4.66)	36.66 (14.90)	0.869[a]
	30 mins Median (IQR)	37.45 (13.22)	31.44 (22.89)	0.496[b]
	60 mins Mean (SD)	36.44 (7.27)	37.77 (12.28)	0.772[a]
Sympathetic (%)	**10 mins Mean (SD)**	13.34 (3.88)	13.60 (5.13)	0.899[a]
	30 mins Mean (SD)	13.18 (5.68)	15.68 (6.67)	0.379[a]
	60 mins Mean (SD)	13.94 (5.89)	13.92 (5.25)	0.993[a]
Endothelial (%)	**10 mins Mean (SD)**	16.69 (5.00)	17.29 (9.00)	0.856[a]
	30 mins Mean (SD)	16.21 (6.59)	15.78 (6.03)	0.882[a]
	60 mins Mean (SD)	16.03 (7.03)	15.67 (8.42)	0.919[a]
Non-endothelial (%)	**10 mins Mean (SD)**	11.29 (5.15)	12.14 (10.83)	0.826[a]
	30 mins Mean (SD)	8.01 (3.29)	10.55 (4.75)	0.183[a]
	60 mins Median (IQR)	9.13 (5.50)	7.54 (4.76)	0.631[b]

6.3.4.2 Males and Females

Sub-group analysis of wavelet analysis data was carried out in males and females. Wavelet analysis was carried out on the FLPI traces collected from male and female participants at baseline, during INP and after one INP session. Percentage contribution of each component was recorded. When comparing baseline to after one INP session in both groups, no significant differences in percentage contribution of any of the six components was recorded. In both groups at both time points, the greatest contribution was from the endothelial component. When comparing between groups, no significant differences for any of the components at either time point was recorded (Table 6.3.10). During INP application, the highest contributing component in both groups was the myogenic component across all three time points. No significant changes in contribution of each component were recorded between the three time points in controls and cold hand volunteers. No significant between group differences were recorded for any of the three time points (Table 6.3.11).

Table 6.3.10: ***Between group comparisons for each component in males and females.*** Measurements obtained from male volunteers and female volunteers at baseline and after one INP session were compared. Normally distributed data was compared using independent samples t-test (a). Non-normally distributed data was compared using Mann-Whitney U test (b). IQR = interquartile range, SD = standard deviation.

		Males	Females	p value
Cardiac (%)	**Before Mean (SD)**	1.65 (0.81)	1.41 (0.79)	0.516[a]
	After Median (IQR)	1.19 (1.22)	1.37 (0.71)	0.877[b]
Respiratory (%)	**Before Median (IQR)**	6.42 (2.78)	4.86 (3.04)	0.217[b]
	After Mean (SD)	5.21 (2.16)	5.31 (1.72)	0.916[a]
Myogenic (%)	**Before Mean (SD)**	16.92 (4.21)	15.30 (6.63)	0.512[a]
	After Median (IQR)	16.32 (6.15)	15.35 (8.64)	0.877[b]
Sympathetic (%)	**Before Mean (SD)**	23.10 (4.02)	23.97 (3.86)	0.639[a]
	After Mean (SD)	22.80 (2.53)	23.65 (2.96)	0.500[a]
Endothelial (%)	**Before Mean (SD)**	32.47 (6.91)	32.15 (6.30)	0.919[a]
	After Median (IQR)	32.23 (6.10)	29.48 (8.16)	0.939[b]
Non-endothelial (%)	**Before Mean (SD)**	19.67 (4.87)	22.12 (5.41)	0.306[a]
	After Mean (SD)	23.08 (5.26)	21.75 (6.98)	0.631[a]

Table 6.3.11: ***Between group comparisons for each component during INP application in males and females.*** Measurements obtained from female volunteers and male volunteers during INP application were compared. Normally distributed data was compared using independent samples t-test (a). Non-normally distributed data was compared using Mann-Whitney U test (b). IQR = interquartile range, SD = standard deviation.

		Males	Females	p value
Cardiac (%)	**10 mins Mean (SD)**	1.26 (0.39)	1.07 (0.22)	0.218[a]
	30 mins Mean (SD)	1.34 (0.38)	1.19 (0.21)	0.325[a]
	60 mins Median (IQR)	1.26 (0.22)	1.39 (0.44)	0.908[b]
Respiratory (%)	**10 mins Mean (SD)**	21.36 (8.25)	18.98 (5.61)	0.486[a]
	30 mins Mean (SD)	21.49 (6.68)	20.64 (6.22)	0.778[a]
	60 mins Median (IQR)	22.27 (7.27)	23.92 (12.24)	0.758[b]
Myogenic (%)	**10 mins Mean (SD)**	34.86 (9.58)	38.31 (12.70)	0.497[a]
	30 mins Mean (SD)	36.28 (7.67)	39.01 (10.74)	0.513[a]
	60 mins Mean (SD)	36.50 (8.97)	38.01 (11.62)	0.746[a]
Sympathetic (%)	**10 mins Mean (SD)**	13.43 (4.11)	13.53 (5.17)	0.962[a]
	30 mins Mean (SD)	14.65 (6.11)	14.11 (5.85)	0.855[a]
	60 mins Mean (SD)	14.02 (4.80)	13.78 (6.62)	0.924[a]
Endothelial (%)	**10 mins Mean (SD)**	16.22 (6.36)	18.13 (8.39)	0.569[a]
	30 mins Mean (SD)	15.89 (6.18)	16.16 (6.53)	0.926[a]
	60 mins Mean (SD)	15.66 (7.69)	16.13 (7.85)	0.896[a]
Non-endothelial (%)	**10 mins Median (IQR)**	9.08 (12.26)	9.70 (11.67)	0.643[b]
	30 mins Mean (SD)	9.54 (4.34)	8.89 (4.21)	0.329[a]
	60 mins Median (IQR)	7.54 (2.66)	9.23 (7.22)	0.700[b]

6.3.5 Summary of findings

- 25 control volunteers and 25 cold hand volunteers were recruited to the cold hand study. 16 females and 9 males were recruited to each group.
- AIx decreased in controls but increased in cold hand volunteers post-INP versus baseline however no changes were significant. Meanwhile, PWV remained consistent in both groups.
- Forearm microvascular blood flow and skin temperature remained consistent between time points in both groups.
- No changes in endothelial function were recorded in either controls or cold hand volunteers after INP application.
- During INP application, no significant variations in hand or fingertip microvascular blood perfusion was recorded in either group.
- Cold hand males had a significantly lower weight and BMI than control males
- Cold hand females had a significantly lower weight than control females

Chapter 7: Discussion

It is clear INP is effective in reducing pain and improving walking distance in some PAD patients as demonstrated by multiple studies. Furthermore, a possible application of INP in further vascular conditions such as Raynaud's phenomenon could have potential. However, the mechanisms behind these clinical benefits have never been uncovered. Several mechanisms have been postulated including activation of the endothelium and contribution of vasoactive chemicals resulting in prolonged increases in blood flow which translate to the clinical benefits exhibited by patients such as wound healing, decreased pain and increased walking capacity. The overall aim of this study was to gain a better understanding of the physiological mechanisms behind INP. The primary objective was to investigate the effect of INP application on vascular function which was achieved through assessment of endothelial function by FLPI (laser Doppler) alongside iontophoresis of ACh and SNP, assessment of arterial stiffness and evaluation of circulatory inflammatory and oxidative stress biomarkers. Further aims of this study included investigating the short and long-term effects as well as local and systemic effects of INP on vascular function. This study provides data regarding the effects of INP on validated surrogate markers of vascular health. This included assessing the peripheral microcirculation which is directly correlated to the central microcirculation and can reflect the general health of the cardiovascular system. Arterial stiffness assessment allows for exploration into the effects of INP on central macrocirculation. Finally, biomarker evaluation allows for the investigation of systemic effects of INP. This study assessed the effects of INP on three different groups: healthy volunteers, PAD patients and individuals exhibiting Raynaud's-like symptoms.

This study was novel in several aspects. This was the first study to:

- Investigate the effects of repeated INP application on healthy physiology
- Evaluate the effects of INP on microvascular endothelial function
- Investigate the short- and long-term effects of INP on vascular function
- Investigate the local and systemic effects of INP
- Investigate the effects of INP as delivered by Flow-Ox on vascular function in individuals with Raynaud's-like symptoms

7.1 The effects of INP on vascular function in healthy volunteers

7.1.1 Summary of key findings

The main findings on the effects in INP in healthy volunteers were:

- AIx and AIx75 significantly decreased after one INP session
- No changes in endothelial function were observed after single or repeated INP sessions compared to baseline
- INP application to the foot of healthy volunteers has no systemic effects on microvascular endothelial function
- Foot blood perfusion and skin temperature both significantly decreased after one INP session
- There were no differences in perfusion or vascular function assessments between the active and placebo devices
- No significant changes in inflammatory or oxidative stress blood borne biomarkers was recorded after repeated INP application, versus baseline.

7.1.2 Interpretation of results

PWV decreased insignificantly after one INP session compared to baseline however increased significantly after repeated INP compared to after one INP session but not compared to baseline (Table 6.1.3). As previously described, with every 1m/s increase in PWV, there is a corresponding 14% increase in cardiovascular risk (Vlachopoulous et al 2010). As only a 0.29% difference in PWV was recorded, this change may be considered clinically non-significant. AIx significantly decreased after one INP session which could be due to the significant changes in diastolic blood pressure and heart rate which occurred as it is well known that AIx is influenced by blood pressure and heart rate (Wilkinson et al 2000). However, upon adjustment for heart rate, a significant decrease in AIx75 was also observed indicating that the difference cannot be due to changing heart rate and therefore may be attributed to a change in peripheral resistance.

When interpreting percentage change in perfusion values it is important to take baseline perfusion values into consideration as a fluctuation in baseline measurements can significantly impact the percentage change in perfusion value. For example, an improvement in percentage change in perfusion in response to ACh may be evident after one INP session, compared to baseline however this may be due to baseline ACh perfusion being lower after one INP session, compared to baseline while peak perfusion remains constant across both time points. In healthy volunteers, an increase in ACh percentage change in perfusion was recorded after one INP session compared to baseline however this was not statistically significant and could be attributed to the significant reduction in baseline ACh perfusion, which was recorded after one INP session, compared to baseline. From this, it can be concluded that no change in local microvascular endothelial function was recorded after INP application, versus baseline, in healthy volunteers. Foot blood perfusion significantly reduced after one INP session from baseline however this was accompanied by a significant reduction in foot skin temperature suggesting the fall in perfusion may be temperature related.

With regards to systemic effects of INP, no change in arm endothelial function was observed and upon correlation analysis with foot endothelial function, no significant correlations between the two sites were recorded. From this, it can be concluded that INP application to the foot in healthy volunteers has no systemic effects on microvasculature endothelial function. Further, the fact no changes in inflammatory or oxidative stress blood biomarkers was recorded supports the idea that INP does not induce systemic physiological effects.

In healthy individuals, INP application induced fluctuations in skin blood perfusion in accordance with the negative pressure cycle as seen in Figure 5.2.1. During INP application, blood perfusion increased compared to when INP was not being applied however these differences were not statistically significant. Similarly, previous studies demonstrated fluctuations in arterial blood flow velocity in accordance with the negative pressure cycle (Sundby et al 2016). These changes in perfusion may arise due to transient changes in transmural pressure which occur with application and release of the negative pressure.

The effects of the active INP device, which delivers a negative pressure of -40mmHg, was compared against the placebo device, which delivers a negative pressure of -10mmHg. The

active device has been shown to increase arterial blood flow compared to baseline meanwhile the placebo device showed no change in arterial blood flow (Hoel at al 2019). Neither active nor placebo INP application induced changes in endothelial function. AIx75 significantly decreased post-INP application in the healthy group, versus baseline. This is in line with the larger group of healthy volunteers in which AIx and AIx75 significantly decreased after one INP session compared to baseline. With the placebo device, a small but significant decrease in PWV was observed after INP application compared to baseline although both values were within a healthy range. Foot blood perfusion significantly decreased after one active INP session and after one placebo INP session, compared to baseline. However, this was also observed in the larger group of healthy volunteers with the active device alongside a significant decrease in foot skin temperature, therefore the changes observed here may also be attributed to temperature change. In fact, a small decrease in skin temperature was recorded in both the active and placebo groups however insignificant. During INP application, foot blood perfusion increased non-significantly in the active compared to when no INP was being applied however in the placebo group, perfusion remained relatively consistent before, during and after INP application. As -40mmHg produces greater increases in blood perfusion as the negative pressure is applied than -10mmHg, as seen in the FLPI perfusion traces, this was to be expected. No between-group differences were recorded for any of the time points of INP application suggesting the active device does not significantly increase foot blood perfusion in healthy volunteers before, during or after INP application compared to the placebo device.

7.1.3 Implications of results

In healthy volunteers, the baseline assessments were, as expected, in normal, healthy ranges therefore there was less scope for improvement after INP application. It is possible that differences after INP application were present however were not big or significant compared to baseline due to baseline already being healthy. Although INP application may not be directly beneficial in this group, INP could be used as a non-pharmacological preventative tool to maintain good vascular health in healthy individuals, although whether long-term cardiovascular outcomes are better in the healthy population with INP application, compared to without INP, would require further investigation.

7.2 The effects of INP on vascular function in PAD patients

7.2.1 Summary of key findings

The main findings on the effects of INP in patients were:

- Pain significantly decreased after repeated INP sessions
- Upon sub-group analysis, pain significantly decreased in non-diabetics but not diabetics and in men but not women
- ABPI remained consistent
- Foot microvascular blood perfusion and endothelial function significantly improved after one INP session
- Foot microvascular blood perfusion and endothelial function improved after repeated INP sessions however not significantly
- The only change in arterial stiffness recorded was a significant increase in PWV after repeated INP versus baseline
- No significant changes in inflammatory or oxidative stress blood borne biomarkers was recorded after repeated INP application, versus baseline

7.2.2 Interpretation of results

No significant changes in ABPI were recorded in patients which is consistent with previous findings which recorded no changes in resting or exercise ABPI alongside increased walking capacity with INP treatment (Hoel et al 2021). Pain significantly decreased in patients after repeated INP application compared to baseline as reported by pain score. The pain experienced by PAD patients can be of different natures including ischaemic pain and neuropathic pain. In PAD, ischaemic pain is extremely common due to the lack of oxygen supply to the muscle. Meanwhile, neuropathic pain is often experienced by diabetic patients as hyperglycaemia leads to nerve damage resulting in numbness and loss of sensation. It is likely that, with INP application, the increased blood flow to the area allows for increased oxygen delivery and a reduction in ischaemic pain. Interestingly, upon sub-group analysis, it was revealed that pain significantly decreased in some sub-groups but not others. While pain significantly decreased in non-diabetics, non-smokers, smokers, and males however not in

diabetics or females. It is not surprising that non-diabetics reported a significant reduction in pain while diabetics did not due to the neuropathic pain they experience as well as generally more advanced and complex disease. This information may provide an indication as to which groups may respond best to INP treatment and which groups the therapy should be targeted towards.

In patients tested at baseline and after one INP session, foot microvascular blood perfusion increased significantly after INP application in the absence of any changes in foot skin temperature suggesting cutaneous blood perfusion increased in the area. Blood perfusion increased non-significantly after repeated INP, compared to baseline also in the absence of any skin temperature variation. While one pervious study demonstrated significant increases in macrovascular blood flow velocity during INP application in PAD patients and another reported no changes in macrovascular blood flow following 12 weeks INP treatment in claudicants, this is the first study to demonstrate an increase in microvascular blood perfusion with INP treatment in PAD patients (Sundby et al 2016, Hoel et al 2020). Microvascular blood perfusion is essential for wound healing and while current therapeutic approaches for CLI, including revascularisation, restores macrovascular circulation, these techniques do not improve microvascular circulation or function. Therefore, INP offers an exciting alternative treatment which can increase microvascular perfusion and promote wound healing.

Evidence presented here suggests an improvement in microvascular endothelial function can be achieved after just one INP session. The percentage change in perfusion with ACh infusion increased significantly after one INP session, compared to baseline in the absence of any changes in baseline perfusion values and also in the absence of any perfusion changes in response to SNP. After repeated INP session, endothelial function did improve insignificantly as peak response to ACh and consequently percentage change in perfusion increased versus baseline in the absence of any changes in SNP reactivity. With a larger sample size this figure may become significant as original sample size calculations for this study calculated that n=25 would provide at least 80% power and 5% significance to detect a 20% before and after difference in assessment. These observations suggest INP can improve endothelial function by increasing NO bioavailability and/or production induced by ACh resulting in the release of endogenous NO from endothelial cells. Although endothelial function may improve through several other mediators including endothelial hyperpolarizing

factor and prostacyclin, considering NO is a significant contributor to endothelial function, the assumption that endothelial function improves here by increasing NO bioavailability is not out with the realms of possibility. No previous studies have proven improved endothelial function may be NO mediated however the use of L-NMMA would help to evaluate this.

Arterial stiffness as measured by augmentation index remained consistent between the three time points however a significant increase in PWV was recorded after repeated INP sessions versus baseline. A mean increase of 1m/s was recorded between these timepoints. It is important to note that with every 1m/s increase in PWV, an individual's cardiovascular risk increases by 14% (Vlachopoulos et al 2010). An increase in PWV in patients may be explained by their cardiovascular disease worsening systemically, over the 8 weeks since baseline measurements however progression of disease was not evaluated in this study.

With regards to systemic effects of INP, no changes in inflammatory or oxidative stress blood biomarkers were recorded suggesting INP does not induce systemic physiological effects. These results mirror results obtained in a recent placebo-controlled study of IC patients (Hoel et al 2021c).

7.2.3 Implications of results

This was the first study to demonstrate that INP can improve foot microvascular blood perfusion and endothelial function after just one INP session in CLI patients.

Patients who have used the Flow-Ox device in this study and previous studies have reported feeling their pain decrease after several sessions, not after just one session. Once INP treatment is stopped for even a couple of days, patients report their pain increasing once again. This may be explained by each session of INP increasing microvascular blood perfusion and endothelial function slightly which accumulates to a physical benefit after repeated sessions and then once INP is not delivered, the endothelium is not being activated, microvascular blood perfusion drops, and pain ensues. One possible effect INP may have on the endothelium is repeated bouts of endothelial activation which results in 'priming the endothelium'. This repeated activation of the endothelium would elicit release of vasoactive chemicals and promote all the positive benefits of the endothelium including vasodilation,

immune defence, and angiogenesis as well as prevention of leukocyte adhesion and platelet activation. These effects may persist after INP application however increases in blood perfusion and endothelial function after repeated INP application were insignificant. Similarly, ischaemic preconditioning (IPC) (described previously 3.1.4) can protect tissue against ischaemia-reperfusion injury through repeated short-bout exposure to ischaemic conditions and previous studies have demonstrated IPC can improve endothelial function however whether IPC is beneficial in PAD remains unproven (Manchurov er al 2014, Jones et al 2014, Hansen et al 2019).

As some patients benefit from INP application but others do not, it is important to be able to identify at baseline which patients will respond positively in order to target this treatment to the correct patients. While this study did analyse different factors which may predict the outcome of patient INP treatment, small sample size meant no specific factor was revealed. It is likely that a mix of multiple parameters may predict who is likely to benefit from INP treatment.

7.3 The effects of INP on vascular function in individuals with Raynaud's-like symptoms

7.3.1 Summary of key findings

The main findings on the effects of INP in individuals with Raynaud's-like symptoms were:

- AIx decreased in controls but increased in cold hand volunteers post-INP versus baseline however no changes were significant. Meanwhile, PWV remained consistent in both groups.
- At baseline, AIx was higher in the control group than cold hands group however after INP application, AIx was higher in the cold hands group than controls. However, no statistically significant between-group differences were recorded.
- Forearm microvascular blood flow and skin temperature remained consistent between time points in both groups.
- No changes in endothelial function were recorded in either controls or cold hands volunteers after INP application

- During INP application, no variations in hand or fingertip microvascular blood perfusion was recorded in either group
- Cold hand males had a significantly lower weight and BMI than control males
- Cold hand females had a significantly lower weight than control females

7.3.2 Interpretation of results

After INP application, aortic stiffness in controls but increased in cold hand volunteers while PWV remained consistent. This may be attributed to small changes in blood pressure and heart rate which occurred in both group although no differences were significant. In controls, a small decrease in heart rate, and slight increase in blood pressure after INP application were recorded. In cold hand volunteers, the opposite occurred with a small increase in heart rate and blood pressure decreasing slightly. As previously described, it is known that AIx is heavily influenced by central haemodynamics however, even when corrected for heart rate (AIx75), these trends prevailed. It is also worth noting that AIx is inversely related to height and heart rate. Since, when adjusted for heart rate, the trend in AIx continued, height may be considered as the cause for the difference however no differences in height between-groups were observed. Therefore, these differences cannot be attributed to heart rate or height but may be attributed to a small but insignificant change in blood pressure, which may have come about due to participants being supine for one hour during assessments. The fact that PWV remains consistent, suggests that the changes in AIx observed may be blood pressure induced.

Forearm microvascular blood perfusion did not significantly change after INP application to the arm. While a previous study demonstrated improved macrovascular endothelial function in the arm of control males with one INP session, this study has demonstrated no change in microvascular forearm endothelial function in either healthy physiology or in individuals with cold hypersensitivity after one INP session. INP may improve macrovascular but not microvascular endothelial function due to the microvasculature being particularly damaged by cardiovascular risk factors and even though these studies were conducted in control volunteers, it may require further intervention to improve endothelial function in the microvasculature. There were also no significant between group differences recorded. Even

with gender sub-analysis, no improvements in endothelial function were recorded in males or females.

During INP application, blood perfusion in the hand and fingertips were lower in the cold hands group than the control group however not significantly. Neither perfusion of the hand or fingertips significantly changed across the three time points of INP application in either group. This suggests INP does not induce any changes in hand or fingertip perfusion during INP application which is contrary to the results observed in the foot of healthy volunteers.

As described previously, the prevalence of Raynaud's is significantly higher in females than males, especially in under 30-year-olds. Therefore, analysis of each female and male group was conducted to assess whether any changes occurred in either gender group however no significant between-group differences were recorded in sub-analysis of males or females suggesting gender did not play a role in the results collected in this study.

Overall, the cold hand group were significantly lighter in weight and had a lower BMI than the control group. When split into gender groups, cold hand males were significantly lighter in weight than control males and cold hand males also had a lower BMI. Despite this, no significant differences were recorded between these two male groups for any of the assessments. The same was true in female groups with cold hand females weighing significantly less than control females and no significant between-group differences being recorded. These weight differences may have impacted results as a lower body fat percentage can result in hypersensitivity to the cold meaning the individuals who self-reported Raynaud's-like symptoms could be experiencing these due to lower body fat percentage instead of an underlying vascular condition (Speakman 2018).

7.3.3 Implications of results

This was the first study to evaluate the effects of INP on microvascular function in the forearm and specifically on individuals who experience Raynaud's-like symptoms.

Although previous studies have demonstrated improved increased microvascular finger blood flow in RP patients with negative pressure application, this study did not show any

improvement in microvascular blood perfusion, endothelial function or arterial stiffness in individuals who experience Raynaud's-like symptoms. The discrepancy in results between previous studies and the current study is most likely due to the length of time INP was applied. Previous studies applied INP repeatedly for approximately 4 weeks however this study only applied INP for one hour. Other reasons for different results could be due to the group assessed. Previous studies recruited diagnosed Raynaud's sufferers whereas this study recruited individuals with self-reported cold hypersensitivity which, as they are not medically diagnosed, could be due to several factors including lower body fat, age and gender.

7.4 Wavelet analysis and vasomotion

As described previously (Section 1.2.4), vasomotion refers to the rhythmic oscillations in the arterial wall which are brought about by six regulatory systems: cardiac, respiratory, myogenic, sympathetic, endothelial, and non-endothelial. It is known that endothelial, myogenic, and even respiratory wavelet energies decrease with age and endothelial amplitudes are greater in young women than young men. In diseased states where the microcirculation is damaged, vasomotion is decreased and in patients who exhibit tissue ischaemia, it is possible for no vasomotion to be present (Intaglietta et al 1990).

As vasomotion is more common in the microvessels, particularly those supplying the skin, the perfusion traces collected from participants in this study were analysed and the contribution of each regulatory component assessed. Wavelet analysis allows for the FLPI perfusion signals to be separated into the six different contributing components and from this, we can explore and determine the contribution of each component to the overall perfusion signal giving insight into how the blood vessels are being regulated. In this study, wavelet analysis allowed exploration into which components contribute during INP application and how each component's contribution changes after INP application, compared to baseline measurements. This was the first study to examine the effects of INP on vasomotion and the six regulatory components.

The percentage contribution of each component to the overall signal were presented here. In all participants (healthy, patients and cold hand volunteers), at baseline, the highest

contributing component was the endothelial component and after INP application, the endothelial component remained the highest contributing component. As the endothelial component is due to NO activity and the endothelium is essential for baseline blood perfusion, these results are to be expected however are surprising in the patient group who are expected to have impaired endothelial function. Although the three study groups were very different in characteristics with the PAD patients being both diseased and significantly older than the healthy volunteers and cold hands groups, in this study, the percentage contribution of each component was comparable between groups. This is contrary to the previous work described which suggested certain components change with age, gender, and presence of disease (Intaglietta et al 1990). While no significant differences were observed in either active or placebo healthy volunteer groups before or after INP application, when comparing active and placebo INP during application, many significant differences were recorded which can be explained by the different magnitudes of negative pressure used. During application of active -40mmHg INP, the highest contributing component was the myogenic component however during application of placebo -10mmHg, after 30 and 60 minutes of INP, the highest contributing component was the endothelial component. This may be explained by the fact placebo negative pressure is similar to that of no pressure. The myogenic regulatory component comes about as smooth muscle cells lining the arterial wall contract and relax in response to changes in pressure. The amplitude of this component is known to be increased by exercise (Shiogai et al 2010). INP application is thought to mimic exercise in that it induces blood pooling which could explain the increase in the contribution of the myogenic component to the overall signal during INP application.

To summarise, in this study, wavelet analysis was used to determine the contribution of each regulatory component to the overall perfusion signal collected from the laser Doppler imager before, during and after INP application. Results suggest during INP application contribution of the myogenic component increases however no changes in any of the components were observed after INP application when compared to baseline. Applying INP may elicit repeated increases in myogenic activity which contributes to the changes in blood perfusion observed during INP application.

7.5 Proposed Mechanism of INP action

The overall aim of this study was the uncover the mechanisms of INP. Theoretical mechanisms of INP were discussed in section 3.3 with both a flow and physiological model being considered. In summary, the flow model proposes that upon application of negative pressure there is a decrease in venous pressure which promotes arterial inflow due to the veno-arteriolar gradient (Smyth 1969). Following a few seconds of negative pressure, vascular resistance is elevated and blood flow declines toward baseline (Skagen & Henriksen 1983). This cycle ensues as the INP cycles proceed. Meanwhile the physiological model proposed that during INP application increased levels of shear stress are exerted on the endothelium due to local increases in blood flow which activates the endothelium to release vasoactive chemicals including NO. With repeated INP application, this cycle would ensue. These two models can be related as the flow model accounts for increases in blood flow during the cycle and the physiological model accounts for increased perfusion and endothelial function observed after INP application in PAD patients.

When the results of this study are taken together, a proposed mechanism of INP action can be suggested. When negative pressure is applied, there is increased blood flow through the vessels due to a fall in venous pressure and consequential increase in arterial inflow. This increased flow exerts shear stress on the endothelium which, through mechano-sensing and -transduction, results in vasodilator release and blood vessel dilation which promotes further increased blood flow to the area. This increased blood flow reduces tissue ischaemia thus reducing ischaemic pain felt by patients. Repeated negative pressure bouts repeatedly stimulates and primes the endothelium resulting in processes such as growth factor release thus promoting wound healing. Once INP application is stopped, there is no priming of the endothelium, no increased blood flow to the area, ischaemic pain returns and wound healing ceases. It is likely the effect of INP is short-term due to repeated reperfusion of the area. This is also demonstrated by patients who report a benefit after a short period of use and report their pain returning after a short period of stopping INP treatment (Figure 7.1).

While this proposed model remains possible in diseased physiology, considering the evidence collected in this study, it is unlikely that INP induces any significant changes in microvascular blood perfusion, endothelial function, or arterial stiffness in healthy

volunteers. It may be the case that in diseased physiology, there is greater scope for improvement than in healthy individuals.

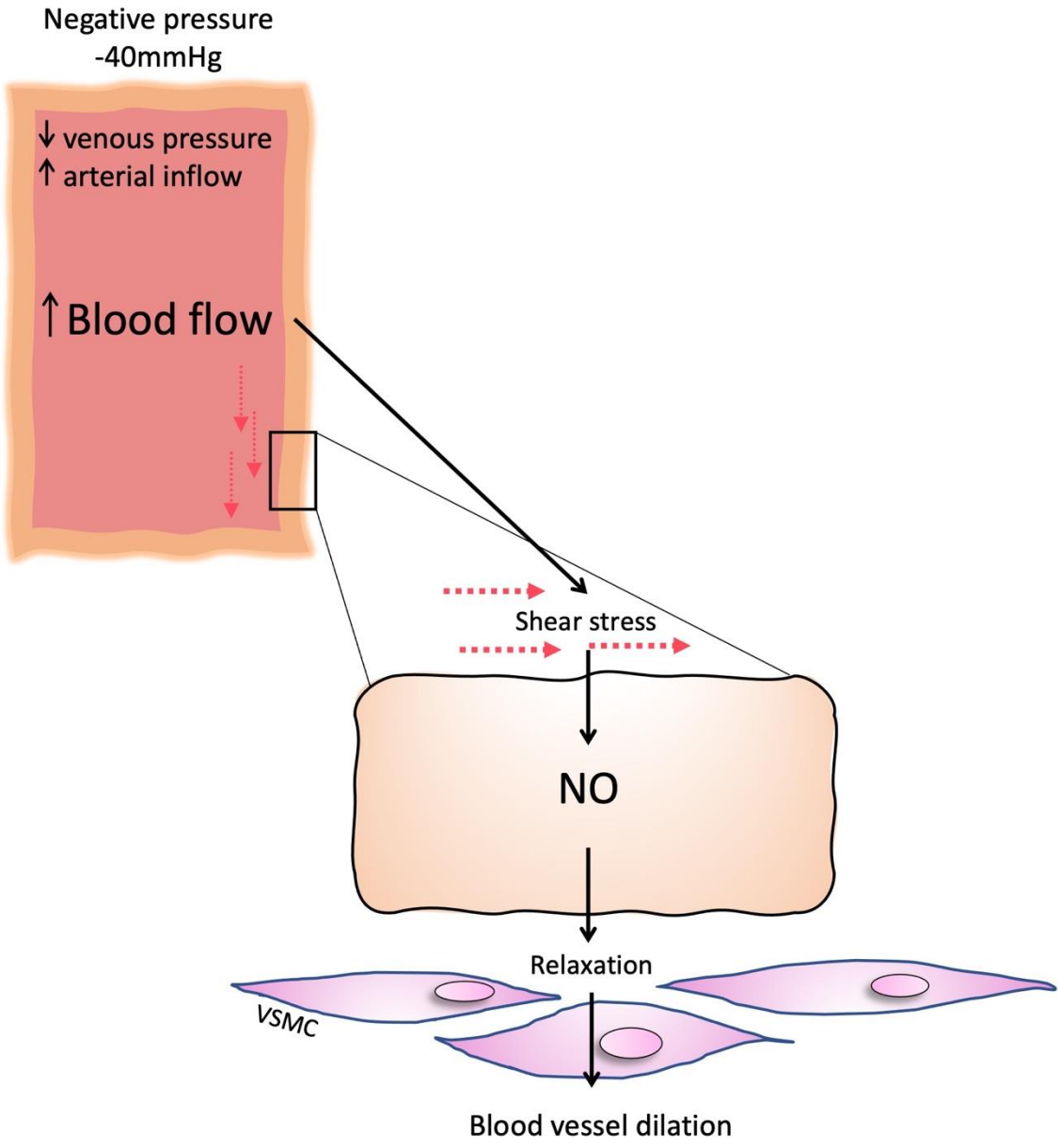

Figure 7.1 ***Proposed potential physiological mechanism of intermittent negative pressure.*** Negative pressure of -40mmHg is applied to the limb which induces a decrease in venous pressure and consequential increase in arterial inflow due to the venoarteriolar gradient. Blood flow in the artery increases which exerts higher levels of shear stress on the endothelium. Shear stress activates intracellular signalling resulting in NO production, VSMC relaxation and vasodilation. Vasodilation further promotes greater arterial inflow. If negative pressure was to be maintained, the venoarteriolar reflex would be initiated which constricts arteries when veins over distend thus leading to a reduction in flow. This is avoided since INP releases negative pressure after 10 seconds. Repeated bouts of negative pressure activate NO production and VSCM relaxation thus promoting long-term vasodilation. VSMC = vascular smooth muscle cell, NO = nitric oxide, INP = intermittent negative pressure.

7.6 Study limitations

The first limitation of this study was the study design as there was no placebo-controlled group. A placebo-controlled single-blinded study was originally planned for patients however, due to the COVID-19 pandemic and recruitment difficulties, the target number of patients could not be achieved and therefore it was decided to use the active device on all patients and not to use the placebo device. A further limitation was that the healthy volunteers and patient group were not age matched. If these groups had been age matched, comparisons between the two groups could have been made and results could have helped to determine if any changes observed were related to age or the PAD condition.

A limitation of the healthy volunteer foot study was the length of time INP was applied. This group used the device for five repeated days only. This was due to the inability of healthy individuals to dedicate a larger amount of time to using the device daily for 2 hours per day. A study exploring the effects of longer-term INP treatment on healthy physiology is warranted since some changes post-repeated INP were observed in patients after using the device for 4-8 weeks.

A limitation of the cold hand's study was the lack of certainty of the participant's self-reported Raynaud's-like symptoms. Participants with cold hands were recruited based on self-reported excessive cold-hypersensitivity and not based on a medical diagnosis of Raynaud's phenomenon. Furthermore, the cold hands study was not blinded, and the researcher knew which participants were healthy, control volunteers and who were cold hands volunteers. In future, a single-blinded placebo-controlled trial is warranted including medically diagnosed Raynaud's patients. Further, both primary and secondary Raynaud's patients could be assessed and compared. Another limitation of the cold hands study was lack of control for the weight of participants. It is well known that individuals who have a lower weight and/or BMI experience hypersensitivity to the cold due to lower body fat percentage and therefore may be the reason behind some cold hands participants reporting excessively cold hands rather than an underlying medical condition such as Raynaud's. A future study including medically diagnosed Raynaud's patients would overcome this.

7.7 Future work

As previously mentioned, a longer-scale healthy volunteer trial may be warranted since this study evaluated the effects of repeated INP in healthy volunteers after just five days of INP application. Although no significant findings were reported in this study, perhaps longer-term INP application may induce changes in vascular function in this group. An 8–12-week study of daily INP application in healthy subjects would allow for comparison with the long-term application INP already recorded in IC patients It may be the case that in healthy volunteers, vascular function is already healthy and cannot be improved upon by INP as there is less scope for improvement however, in this case, INP may be considered as a preventative tool in the maintenance of a healthy microvasculature which could be explored in a longitudinal study.

This study revealed a significant improvement in endothelial function in patients after one INP session compared to baseline. However, due to the small patient numbers achieved, a larger scale placebo-controlled trial investigating vascular function is warranted. As previously described, the original sample size calculation for this study calculated that 25 participants would provide at least 80% power and 5% significance to detect a 20% before and after difference in assessment therefore a study achieving these numbers in all assessments would provide more conclusive, significantly valid results. Having both age-matched healthy and placebo control groups alongside the patient group would allow for comparisons to be made in respect to the effects of INP on patients compared to healthy age-matched individuals. Although INP has now been tested in a variety of PAD patients from intermittent claudication to critical limb ischaemia, it is still unknown why some patients see a benefit and some patients do not. For INP to be an effective treatment modality, the ability to determine if a patient will respond positively to treatment or not is essential. It is still unknown what baseline characteristics of PAD patients may determine their response to INP and what sub-groups of patients will benefit the most from INP treatment and it is likely to be a combination of factors which determine this. Furthermore, a study evaluating the effects of INP treatment on clinical outcome versus standard therapy would provide evidence regarding whether INP treatment could be adopted into standard care.

Blood samples collected in this study from healthy volunteers and patients were used for serum and plasma analysis of blood borne inflammatory and oxidative stress markers. Peripheral blood mononuclear cells (PBMCs) were also extracted from blood samples and stored. A further study could conduct gene expression analysis using such methods as TaqMan gene expression assay to assess expression levels of Nrf2 target genes and therefore evaluate whether INP application can induce any systemic changes in oxidative stress levels.

As the cold hands study included participants with self-reported symptoms, a study including patients with medically diagnosed Raynaud's is warranted. The cold hands study was conducted as a pilot study to gain insight into any changes which may occur in individuals with excessively cold hands. The results warrant a further exploration into vascular function of patients with medically diagnosed Raynaud's Phenomenon. Such study would benefit from having placebo and healthy age, sex and weight matched control groups. Furthermore, investigating the effects of repeated INP application over several weeks on ischaemic attack frequency and severity by means of a patient diary would allow for evaluation as to whether INP could produce clinically meaningful outcomes in these patients as well as comparison with standard therapy such as calcium-channel blockers (CCBs). Conversely, INP may be considered as a preventative tool to prevent attacks. INP application may be considered as a recovery tool which is applied when an ischaemic attach ensues and relieves symptoms. A study investigating the effects of INP on blood flow and pain during an ischaemic attack in Raynaud's patients could evaluate this.

7.8 Conclusions

- INP does not induce any systemic changes in vascular function in healthy or diseased physiology
- Repeated INP application significantly reduces pain in patients with PAD
- INP application may improve local microvascular blood perfusion and endothelial-dependent vasodilation in patients with PAD in the short-term however there does not seem to be any significant long-term effects of INP on vascular function

- INP may elicit increases in shear stress through increased arterial inflow, resulting in increased vasodilator release, VSMC relaxation and vasodilation which further promotes arterial inflow. This action may be confined to while INP is being applied.

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Patient Information Sheet (PAD Group)

Full Study title: **Mechanistic insights into changes in peripheral blood flow following intermittent negative pressure.**

Name of Chief Investigator: Professor Faisel Khan

We would like to invite you to take part in our study which is a part of a PhD research degree of Jody McIntosh in Vascular Medicine under the supervision of Prof Faisel Khan and Mr Stuart Suttie at the University of Dundee. Before you decide if you want to take part, we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have.

Please take time to read this information carefully.

Talk to others about the study if you wish. We will do our best to answer your questions and give you any more information you ask for. You do not have to decide straight away.

What is the purpose of the study?

The purpose of this research study is to see if application of pressure to the leg can improve blood flow.

Peripheral arterial disease (PAD) is a disease of the blood vessels which restricts blood flow to the leg and foot. PAD sufferers may experience lower limb pain, develop hard-to-heal ulcers and have an increased risk of developing further cardiovascular (heart and blood vessel) disease. Ultimately, PAD can lead to lower leg amputation.

Previous studies have shown that applying pressure to the leg has positive effects on blood flow. We wish to see whether pressure application can be helpful in increasing blood flow and reducing pain in PAD patients. If so, this may offer a novel treatment option for these patients and may help reduce the number of leg amputations performed in hospitals.

Who is organising and funding the research?

This clinical research study is sponsored by the University of Dundee and NHS Tayside and funded by Otivio AS, Oslo, Norway. The study was organised by Professor Faisel Khan from the University of Dundee.

Why have I been invited?

This study is looking at patients who have been diagnosed with PAD with ongoing pain. This patient group have been shown to benefit from pressure application in previous studies. We will be asking approximately 80 patients, in Ninewells Hospital, to take part in this study.

Do I have to take part?

No. It is up to you to choose, taking part in this study is entirely up to you. You can choose to take part or choose not to take part. If you choose to take part you can stop the study at any time. You do not have to give a reason for not taking part or for stopping. If you do not want to take part or want to stop the study the medical care you get and your relationship with the medical or nursing staff looking after you will not be affected. If you do decide to take part but later decide to withdraw or one of the research doctors or one of your healthcare team advises you to withdraw from the study, we would like your permission to keep and use the information already collected. If you do not give permission for us to keep and use the information already collected, then it will be removed from the study. This is considered as withdrawal of your consent from participation in this study. It is important that you inform us if this is the case. No further study-related activities will take place.

Will taking part in the study affect my usual care?

No, you will continue to receive your primary care. The study will not affect your current or future treatment at all.

What will happen to me if I take part?

A medical doctor will make sure that it is suitable for you to take part in this study. As pregnant women will not be included in this study, pregnancy testing will be carried out in females prior to taking part. If you are eligible and decide to take part in this study, you will have the opportunity to try the Flow-ox boot in the presence of clinical staff prior to taking it home. At the start of the study, you will randomly be given either an active Flow-Ox boot or placebo (dummy) Flow-Ox boot. The negative pressure will be higher in the active boot compared to the placebo but you should not feel a difference. You will not be told until the end of the study which boot you are given.

We will invite you to the Division of Systems Medicine research department of the Medical School at Level 7, Ninewells Hospital to obtain consent from you prior to performing any study assessments. If you are happy to go ahead, we will proceed to conduct the study assessments.

Study assessments will be performed on up to three separate occasions; at the first visit before intermittent negative pressure (INP), following 1 session of INP and following 4-8 weeks of daily INP.

Blood sample: We will take a 40 ml blood sample (approximately 8 teaspoons) from your arm to measure levels of inflammatory substances within the blood.

We will then perform tests to measure how well your blood vessels work. These tests are non-invasive and will take no more than 1 hour to complete. You will be asked to stay in a lying or seated position while we perform tests.

Laser Imaging: Microvascular (small blood vessel) function will be assessed on your lower leg/foot and arm by using a low-grade laser which measures blood flow of the small vessels under your skin. Two small, circular chambers will be stuck to your skin using adhesive tape, which will be removed after the test. Using a small electric current, a very small amount of two solutions called acetylcholine and sodium nitroprusside will be applied, through your skin into the blood vessels. This area of skin will be scanned with a low-grade laser light to measure blood flow during solution delivery. You might experience some mild tingling sensation but no pain.

Pain Chart: You will be asked to fill out a pain chart. In this form you will be asked to numerically rate your pain on a scale of 0-10 (ranging from no pain (0) to the worst pain they have ever felt (10)).

ABPI Monitoring: An ultrasound probe will be used to hear the sound of blood at your ankle artery and brachial artery at your arm. A cuff will be used to measure pressure at your arm and lower leg. A score will be given at the end depending upon the pressure at your both sites.

Arterial compliance: The elasticity of the blood vessels in your neck, wrist and thigh will be assessed by measuring your blood pressure, and then a small pencil-like device will be placed over your skin to measure your pulse and a cuff will be placed on your arm and thigh.

Flow-ox: The Flow-ox machine is designed to apply pressure to your leg through a pressure chamber. The pressure chamber is shaped like a boot, so you can comfortably insert your leg (Figure 1). This is connected to a control unit. The machine will be given to you to take home along with an experimental plan, so you know when to use it. You will be expected to use the machine for an hour, twice a day for up to 8 weeks. A full description and training will be given beforehand. As this study is new research, we do not plan at present to make this treatment available at the end of the study.

Figure 1: Patient set up with the Flow-ox boot.

You will be asked to be comfortably clothed and seated in your home 20 minutes before using the boot. You can keep any dressings or socks on. You will be asked to place your leg into the Flow-ox boot with the other leg remaining outside the boot. The boot will be sealed below the knee with a rubber seal. Once turned on, the machine will begin to apply pressure and you should remain seated comfortably for the duration. During this time you can remain seated and relax.

The pressure will be continuously monitored within the boot. Pressure will be applied in a cycle of 10 seconds of pressure and 7 seconds of no pressure for an hour. There is a display on the control unit showing time of INP application. After an hour, turn the machine off. The data will automatically be stored and analysed at the end of the study by the study team.

A member of the study team will contact you regularly via phone calls to ensure you are not experiencing any problems using the Flow-ox machine and to answer any questions you have.

At the end of this study the machine will require further testing and research before the treatment can be made available to patients.

We will also collect basic demographic data about you from your clinical notes such as age, gender and details of any operation you have had.

There are no additional requirements and no further assessments.

Expenses and payments

There will be no cost to you for participating in this study. Reimbursements can be made for reasonable travel expenses. Please discuss this with a member of the research team who will provide you with details of reimbursement. Reimbursement of up to £50 can be made at the end of the study.

What if relevant new information becomes available?

Sometimes during the research study, new information is found out about how to care for patients. . For example, changes related to your heart and blood vessels (blood pressure). If this happens, we will tell you about it and discuss with you whether you want to or should continue your participation in this study. If you decide not to carry on, we will make arrangements for your care to continue. If you decide to continue in the study, you will be asked to sign an updated consent form. Also, on receiving new information, we might consider it to be in your best interest to withdraw from the study. If so, we will explain the reasons and arrange for your care to continue.

What are the alternatives for treatment?

There are no alternatives, if you do not take part in the study you will not be disadvantaged in any way.

What are the possible disadvantages and risks of taking part?

You may experience some discomfort, pain or bruising from blood tests but extra care will be taken to minimise this. All other study assessments are non-invasive however sometimes might experience some mild tingling sensation but no pain. We do not anticipate any additional risks.

What are the possible benefits of taking part?

We do not anticipate any direct benefit for patients participating in this study. Information from this study may help people with peripheral blood vessel disease in the future.

What happens when the research study stops?

You will not be required to have any further involvement.

What if there is a problem?

If you are concerned about your participation in the study you have the right to discuss your concern with a researcher involved in carrying out the study or a doctor involved in your care.

If you have a complaint about your participation in the study first of all you should talk to a researcher involved in the trial/study. You can also make a formal complaint. You can make a complaint to a senior member of the research team or to the Complaints Officer for NHS Tayside:

The Complaints and Feedback Team,
Ninewells Hospital,
Dundee,
DD1 9SY,
Telephone 0800 027 5507,
email feedback.tayside@nhs.net

If you think you have come to harm due to taking part in the study there are not any automatic arrangements to get financial compensation. You might have the right to make a claim for compensation. If you wish to make a claim, you should think about getting independent legal advice but you might have to pay for your legal costs.

The University of Dundee and Tayside Health Board are Co-Sponsoring the study. The University of Dundee has a policy of public liability insurance which provides legal liability to cover damages, costs and expenses of claims.

Tayside Health Board is a member of the NHS Scotland Clinical Negligence and Other Risks Insurance Scheme (CNORIS) which gives legal liability cover of NHS Tayside for this study.

As the study involves University of Dundee staff carrying out clinical research on NHS Tayside patients, these staff hold honorary contracts with Tayside Health Board. This means they will be covered under Tayside's membership of the CNORIS scheme.

If you apply for health, life, travel or income protection insurance you may be asked questions about your health. These questions might include questions about any medical conditions you have or have had in the past. You might also be asked about taking part in this study. We do not expect that taking part in the study will adversely affect your ability to buy insurance. Some insurers may use this information to limit the amount of cover, apply exclusions or increase the cost of insurance. Your insurer may take in to account any medical conditions you have, including any which are diagnosed as part of a research study, when deciding whether to offer insurance to you.

What if I change my mind about taking part or continuing in the study?

Your clinical care will not be affected in anyway. You would be withdrawn from the study. Any data or samples already collected with your consent would be retained and used in the study, unless you request your data to

be withdrawn from the study. We would not collect any further data or samples and no other research procedures would be carried out.

Will my taking part in the study be kept confidential?

Identifiable information about you and the information collected about you during the study will be stored by the University of Dundee and NHS Tayside. Specified members of the research team will have access to this information and it may be shared outside the research team where it is needed to carry out for this study. Your confidential information collected for this study may be used in ethically approved medical research in the future. Any information which identifies you will be removed before it is shared.

Your identifiable information and coded study information will be stored securely on a password-protected database(s) in the University of Dundee. Specified members of the data management team will also have access to your identifiable information to manage your information and maintain the database.

Your information will be kept securely for 15 years after the end of the study. After 15 years it will be destroyed. If you would like to be informed about future studies that you might be interested to participate we will ask you to sign a consent to allow us to hold your contact details.

We will ask your permission to tell your GP that you are taking part in this study.

Information which identifies you will not be published or shared.

All and any identifiable information about you will be removed and fully anonymised before study data is shared with other researchers in the UK/EU/other.

Involvement of the General Practitioner/Family doctor (GP).

We will inform your GP that you have agreed to take part in this study and if there is an incidental findings related to your health.

What will happen to any samples I give?

Blood samples will be collected as part of this study for different tests. Most of these tests will be performed immediately. If you allow us to take these they may be stored for up to 15 years to allow us to do new tests in the future. You will not be able to be identified personally however.

What will happen to the results of the research study?

When the results become available they will be submitted to medical journals where they will be considered for publication. Patient data used in reported results will be fully anonymised. The final results will also be

submitted to national and international medical conferences where they will be considered for presentation. The study doctors may take part in events at their institutions to inform the public about their ongoing research and about results from this and other studies. The results may also form part of a research degree.

You will not be identified in any report or publication.

If you would like a copy of the results, please ask your study doctor.

Who has reviewed the study?

The East of Scotland Research Ethics Service, which has responsibility for scrutinising all proposals for medical research on humans in Tayside, has examined the proposal and has raised no objections from the point of view of research ethics. It is a requirement that your records in this research, together with any relevant medical records, be made available for scrutiny by monitors from The University of Dundee and NHS Tayside, whose role is to check that research is properly conducted and the interests of those taking part are adequately protected.

Further information and contact details

Please feel free to contact the study doctors if you need any further information:

Miss Jody McIntosh
PhD student
Division of Systems Medicine, University of Dundee
Ninewells Hospital and Medical School
Tel: 01382 385711
Email: j.y.mcintosh@dundee.ac.uk

Professor Faisel Khan
Professor of Cardiovascular Sciences
Division of Systems Medicine, University of Dundee
Ninewells Hospital and Medical School
Tel: 01382 383531
E-mail: f.khan@dundee.ac.uk

Mr Stuart Suttie
Consultant Vascular Surgeon
Ward 12, Ninewells Hospital
Tel: 01382-660111 Ext: 33332
E-mail: stuartsuttie@nhs.net

Mr John Nagy
Consultant Vascular Surgeon
Ward 12, Ninewells Hospital
Tel: 01382-660111 Ext: 33332
E-mail: jnagy@nhs.net

You may also discuss the study with a contact who is not directly involved with the study (Dr Graham Guthrie).

Dr Graeme Guthrie
Specialty Registrar
Ward 12, Ninewells Hospital
Tel: 01382 633889
E-mail: graemeguthrie@doctors.org.uk

Thank you for taking time to read this information and for considering taking part in this study.

If you are interested in taking part or would like more information please contact Jody McIntosh using the contact details above.
You can contact us Monday – Friday between 9:00-17:00.

Data Protection Privacy Notice

How will personal information be used?

We will only use your personal information to carry out this study.

The University of Dundee and NHS Tayside are the sponsors for this study based in the United Kingdom. We will be using information from you and your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. University of Dundee will keep identifiable information about you for 15 years after the study has finished.

Your rights to access, change or move your information are limited, as we need to manage your information in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To protect your rights, we will use the minimum amount of information which is personably identifiable as possible.

DIRECTLY COLLECTED DATA

NHS Tayside and University of Dundee will use your name, NHS number and contact details to contact you about the study. They will use this information to make sure that relevant information about the study is recorded for your care and to check the quality of the study. Staff from University of Dundee/NHS Tayside and regulatory organisations may look at your medical and research records to check the accuracy of the research study. Ninewells Hospital will pass these details to University of Dundee/NHS Tayside along with the information collected from you and your medical records. The only people in University of Dundee/NHS Tayside who will have access to information that identifies you will be people who need to contact you to take part in the study. The people who analyse the information will not be able to identify you and will not be able to find out your name, NHS number or contact details.

University of Dundee will keep identifiable information about you from this study for 15 years after the study has finished.

INDIRECTLY AND DIRECTLY COLLECTED DATA

University of Dundee/NHS Tayside will collect information about you for this study from medical records. This information will include your name, NHS number, contact details and health information. Health information which is regarded as a special category of information. We will only use this information to conduct this study.

Lawful reason for using your information

It is lawful for the University/NHS Tayside to use your personal data for the purposes of this study. The legal reason for using your information is that using it is necessary for the research which is carried out in the public interest.

It is lawful for the University/NHS Tayside to use your sensitive personal data (if applicable) for the purposes of this study. The reason we use sensitive personal information such as data concerning health is that using it is necessary for scientific research purposes. Legally we must ensure we have technical and organisational processes in place to respect your rights when we use your information.

You can find out more about how we will use your information at http://www.ahspartnership.org.uk/tasc/for-the-public/how-we-use-your-information and https://www.dundee.ac.uk/information-governance/dataprotection/ and at http://www.nhstayside.scot.nhs.uk/YourRights/PROD_298457/index.htm

Or by contacting Research Governance, Tayside Medical Science Centre (TASC), 01382 383900 email tascgovernance@dundee.ac.uk

If you wish to complain about the use of your information please email dataprotection@dundee.ac.uk or, informationgovernance.tayside@nhs.net or, you may wish to contact the Information Commissioner's Office.

Healthy Volunteer Information Sheet

Full Study title: **Mechanistic insights into changes in peripheral blood flow following intermittent negative pressure.**

Name of Chief Investigator: Professor Faisel Khan

We would like to invite you to take part in our study which is part of a PhD research degree of Jody McIntosh in Vascular Medicine under the supervision of Prof Faisel Khan and Mr Stuart Suttie at the University of Dundee. Before you decide if you want to take part, we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have.

Please take time to read this information carefully.

Talk to others about the study if you wish. We will do our best to answer your questions and give you any more information you ask for. You do not have to decide straight away.

What is the purpose of the study?

The purpose of this research study is to see if application of pressure to the leg can improve blood flow.

Peripheral arterial disease (PAD) is a disease of the blood vessels which restricts blood flow to the leg and foot. PAD sufferers may experience lower limb pain, develop hard-to-heal ulcers and have an increased risk of developing further cardiovascular (heart and blood vessel) disease. Ultimately, PAD can lead to lower leg amputation.

Previous studies have shown that applying pressure to the leg has positive effects on blood flow. We wish to see whether pressure application can be helpful in increasing blood flow and reducing pain in PAD patients. If so, this may offer a novel treatment option for these patients and may help reduce the number of leg amputations performed in hospitals.

Who is organising and funding the research?

This clinical research study is sponsored by the University of Dundee and NHS Tayside and funded by Otivio AS, Oslo, Norway. The study was organised by Prof Faisel Khan from the University of Dundee.

Why have I been invited?

You are being invited because you might fit the criteria for one of the groups in the study. In order for us to know if any discoveries we make in PAD patients are significant, we also require to test healthy volunteers like you to compare against. We will be asking approximately 40 healthy people to take part in this study.

Do I have to take part?

No. It is up to you to choose, taking part in this study is entirely up to you. You can choose to take part or choose not to take part. If you choose to take part you can stop the study at any time. You do not have to give a reason for not taking part or for stopping. If you do decide to take part but later decide to withdraw or one of the research doctors advises you to withdraw from the study, we would like your permission to keep and use the information already collected. If you do

not give permission for us to keep and use the information already collected then it will be removed from the study. This is considered as withdrawal of your consent from participation in this study. It is important that you inform us if this is the case. No further study-related activities will take place.

What will happen to me if I take part?

A member of the research team will make sure that it is suitable for you to take part in this study. As pregnant women will not be included in this study, pregnancy testing will be carried out in females prior to taking part.

If you are eligible and decide to take part in this study you will have the opportunity to try the Flow-ox boot prior to taking it home. We will invite you to the Division of Systems Medicine research department of the Medical School at Level 7, Ninewells Hospital to obtain consent from you prior to performing any study assessments. If you are happy to go ahead we will proceed to conduct the study assessments.

Study assessments will be performed on up to three separate visits; at the first visit before Intermittent negative pressure (INP), following 1 session of INP and following up to 5 days of daily INP.

We will take a 40 ml blood sample (approximately 8 teaspoons) from your arm to measure levels of inflammatory substances within the blood. We will then perform tests to measure how well your blood vessels work. These tests are non-invasive and will take no more than 1 hour to complete. You will be asked to stay in a lying or seated position while we perform tests.

Laser Imaging: Microvascular (small blood vessel) function will be assessed on your lower leg/foot and arm by using a low-grade laser which measures blood flow of the small vessels under your skin. Two small, circular chambers will be stuck to your skin using adhesive tape, which will be removed after the test. Using a small electric current, a very small amount of two solutions called acetylcholine and sodium nitroprusside will be applied, through your skin into the blood vessels. This area of skin will be scanned with a low-grade laser light to measure blood flow during solution delivery. You might experience some mild tingling sensation but no pain.

ABPI Monitoring: An ultrasound probe will be used to hear the sound of blood at your ankle artery and brachial artery at your arm. A cuff will be used to measure pressure at your arm and lower leg. A score will be given at the end depending upon the pressure at both sites.

Arterial compliance: The elasticity of the blood vessels in your neck, arm and thigh will be assessed by measuring your blood pressure, and then a small pencil-like device will be placed over your skin to measure your pulse and a cuff will be placed on your arm and thigh.

Flow-ox: The Flow-ox machine is designed to apply intermittent negative pressure (INP) to your leg through a pressure chamber. The pressure chamber is shaped like a boot, so you can comfortably insert your leg (Figure 1). This is connected to a control unit. The machine will be given to you to take home along with an experimental plan, so you know when to use it. You will be expected to use the machine for around an hour, twice a day for up to 5 days. A full description and training will be given beforehand.

Figure 1: Patient set up with the Flow-ox boot and control unit.

You will be asked to be comfortably clothed and seated in your home 20 minutes before using the boot. You can keep socks on. You will be asked to place your leg into the Flow-ox boot with the other leg remaining outside the boot. The boot will be sealed below the knee with a rubber seal. Once turned on, the machine will begin to apply pressure and you should remain seated comfortably for the duration. During this time you can remain seated and relax.

The pressure will be continuously monitored within the boot. Pressure will be applied in a cycle of 10 seconds of negative pressure and 7 seconds of no pressure for an hour. There is a display on the control unit showing time of INP application. After an hour, turn the machine off. The data will automatically be stored and analysed at the end of the study by the study team.

A member of the study team will contact you regularly via phone calls to ensure you are not experiencing any problems using the Flow-ox machine and to answer any questions you have.

We will also collect basic demographic data about you such as age and gender.

There are no additional requirements and no further assessments.

Expenses and payments

There will be no cost to you for participating in this study. Reimbursements can be made for reasonable travel expenses. Please discuss this with a member of the research team who will provide you with details of reimbursement. Reimbursement of up to £50 can be made at the end of the study.

What if relevant new information becomes available?

We will tell you about any new information that may affect your participation in the study. For example, changes related to your heart and blood vessels (eg. blood pressure). You can then decide if you want to continue study intervention and other study-related activities.

What are the possible disadvantages and risks of taking part?

You may experience some discomfort, pain or bruising from blood tests but extra care will be taken to minimise this. All other study assessments are non-invasive however you might experience some mild tingling sensation but no pain. We do not anticipate any additional risks.

What are the possible benefits of taking part?

We do not anticipate any direct benefit for healthy volunteers participating in this study. Information from this study may help people with peripheral blood vessel disease in the future.

What happens when the research study stops?

You will not be required to have any further involvement.

What if there is a problem?

If you are concerned about your participation in the study you have the right to discuss your concern with a researcher involved in carrying out the study or a doctor involved in your care.

If you have a complaint about your participation in the study first of all you should talk to a researcher involved in the study. You can also make a formal complaint. You can make a complaint to a senior member of the research team or to the Complaints Officer for NHS Tayside:

The Complaints and Feedback Team,
Ninewells Hospital,
Dundee,
DD1 9SY,
Telephone: 0800 027 5507,
Email: feedback.tayside@nhs.net

If you think you have come to harm due to taking part in the study there are not any automatic arrangements to get financial compensation. You might have the right to make a claim for compensation. If you wish to make a claim, you should think about getting independent legal advice but you might have to pay for your legal costs.

The University of Dundee and Tayside Health Board are Co-Sponsoring the study. The University of Dundee has a policy of public liability insurance which provides legal liability to cover damages, costs and expenses of claims.

Tayside Health Board is a member of the NHS Scotland Clinical Negligence and Other Risks Insurance Scheme (CNORIS) which gives legal liability cover of NHS Tayside for this study.

As the study involves University of Dundee staff carrying out clinical research on NHS Tayside patients, these staff hold honorary contracts with Tayside Health Board. This means they will be covered under Tayside's membership of the CNORIS scheme.

If you apply for health, life, travel or income protection insurance you may be asked questions about your health. These questions might include questions about any medical conditions you have or have had in the past. You might also be asked about taking part in this study. We do not expect that taking part in the study will adversely affect your ability to buy insurance. Some insurers may use this information to limit the amount of cover, apply exclusions or increase the cost of insurance. Your insurer may take in to account any medical conditions you have, including any which are diagnosed as part of a research study, when deciding whether to offer insurance to you.

What if I change my mind about taking part or continuing in the study?

You would be withdrawn from the study. Any data or samples already collected with your consent would be retained and used in the study, unless you choose to withdraw your data from the study. We would not collect any further data or samples and no other research procedures would be carried out.

Will my taking part in the study be kept confidential?

Identifiable information about you and the information collected about you during the study will be stored by the University of Dundee and NHS Tayside. Specified members of the research team will have access to this information and it may be shared outside the research team where it is needed to carry out for this study. Your confidential information collected for this study may be used in ethically approved medical research in the future. Any information which identifies you will be removed before it is shared.

Your identifiable information and coded study information will be stored securely on a password-protected database(s) in the University of Dundee. Specified members of the data management team will also have access to your identifiable information to manage your information and maintain the database.

Your information will be kept securely for 15 years after the end of the study. After 15 years it will be destroyed. If you would like to be informed about future studies that you might be interested to participate we will ask you to sign a consent to allow us to hold your contact details.

Information which identifies you will not be published or shared.

All and any identifiable information about you will be removed and fully anonymised before study data is shared with other researchers in the UK/EU/other.

What will happen to any samples I give?

Blood samples will be collected as part of this study for different tests. Most of these tests will be performed immediately. If you allow us to take these they may be stored for up to 15 years to allow us to do new tests in the future. You will not be able to be identified personally however.

What will happen to the results of the research study?

When the results become available they will be submitted to medical journals where they will be considered for publication. Patient data used in reported results will be fully anonymised. The final results will also be submitted to national and international medical conferences where they will be considered for presentation. The study doctors may

take part in events at their institutions to inform the public about their ongoing research and about results from this and other studies. The results may also form part of a research degree.

You will not be identified in any report or publication.

If you would like a copy of the results, please ask your study doctor.

Who has reviewed the study?

The East of Scotland Research Ethics Service, which has responsibility for scrutinising all proposals for medical research on humans in Tayside, has examined the proposal and has raised no objections from the point of view of research ethics. It is a requirement that your records in this research, together with any relevant medical records, be made available for scrutiny by monitors from The University of Dundee and NHS Tayside, whose role is to check that research is properly conducted and the interests of those taking part are adequately protected.

Further information and contact details

Please feel free to contact the study doctors if you need any further information:

Miss Jody McIntosh
PhD student
Division of Systems Medicine, University of Dundee
Ninewells Hospital and Medical School
Tel: 01382 383479
E-mail: j.y.mcintosh@dundee.ac.uk

Professor Faisel Khan
Professor of Cardiovascular Sciences
Division of Systems Medicine, University of Dundee
Ninewells Hospital and Medical School
Tel: 01382 383531
E-mail: f.khan@dundee.ac.uk

Mr Stuart Suttie
Consultant Vascular Surgeon
Ward 12, Ninewells Hospital
Tel: 01382-660111 Ext: 33332
E-mail: stuartsuttie@nhs.net

Mr John Nagy
Consultant Vascular Surgeon
Ward 12, Ninewells Hospital
Tel: 01382-660111 Ext: 33332
E-mail: jnagy@nhs.net

You may also discuss the study with a contact who is not directly involved with the study (Dr Graham Guthrie).

Dr Graeme Guthrie
Specialty Registrar
Ward 12, Ninewells Hospital
Tel: 01382 633889
E-mail: graemeguthrie@doctors.org.uk

Thank you for taking time to read this information and for considering taking part in this study.

If you would like more information or want to ask questions about the study please contact the study team using the contact details above.

You can contact us Monday – Friday between 09:00-17:00.

Data Protection Privacy Notice

How will personal information be used?

We will only use your personal information to carry out this study.

The University of Dundee and NHS Tayside are the sponsors for this study based in the United Kingdom. We will be using information from you and your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. University of Dundee will keep identifiable information about you for 15 years after the study has finished.

Your rights to access, change or move your information are limited, as we need to manage your information in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To protect your rights, we will use the minimum amount of information which is personably identifiable as possible.

DIRECTLY COLLECTED DATA

University of Dundee will use your name, NHS number, health information and contact details to contact you about the study. They will use this information to make sure that relevant information about the study is recorded for your care and to check the quality of the study. Staff from University of Dundee and regulatory organisation may look at your research records to check the accuracy of the research study. The only people in University of Dundee who will have access to information that identifies you will be people who need to contact you to take part in the study. The people who analyse the information will not be able to identify you and will not be able to find out your name, NHS number or contact details.

University of Dundee will keep identifiable information about you from this study for 15 years after the study has finished.

Lawful reason for using your information

It is lawful for the University/NHS Tayside to use your personal data for the purposes of this study. The legal reason for using your information is that using it is necessary for the research which is carried out in the public interest.

It is lawful for the University/NHS Tayside to use your sensitive personal data (if applicable) for the purposes of this study. The reason we use sensitive personal information such as data concerning health is that using it is necessary for scientific research purposes. Legally we must ensure we have technical and organisational processes in place to respect your rights when we use your information.

You can find out more about how we will use your information at http://www.ahspartnership.org.uk/tasc/for-the-public/how-we-use-your-information and https://www.dundee.ac.uk/information-governance/dataprotection/ and at http://www.nhstayside.scot.nhs.uk/YourRights/PROD_298457/index.htm

Or by contacting Research Governance, Tayside Medical Science Centre (TASC), 01382 383900 email tascgovernance@dundee.ac.uk

If you wish to complain about the use of your information please email dataprotection@dundee.ac.uk or, informationgovernance.tayside@nhs.net or, you may wish to contact the Information Commissioner's Office.

Cold Hands Volunteer Information Sheet

Full Study Title: The potential role of intermittent negative pressure (INP) in improving peripheral blood flow in individuals with excessive vasoconstriction

Name of Chief Investigator: Professor Faisel Khan

We would like to invite you to take part in our study, which is part of the PhD research degree of Jody McIntosh in Vascular Medicine, under the supervision of Professor Faisel Khan at the University of Dundee. Before you take part, we invite you to read and understand why our research is being done and what is involved. One of our team will go through the information and can answer any questions you have.

Please take the time to read through this information carefully.

You can talk to other people about the study if you wish and we will do our best to answer your questions. We can also give you any additional information that you would like. You do not have to decide straight away

What is the purpose of the study?

The purpose of this research study is to see if application of pressure to the arm can improve blood flow. We will be looking at those who have those with what we call 'excessive vasoconstriction'.

Excessive vasoconstriction occurs when cold or changes in temperature result in reversible change in colour, usually of the fingers. Colour changes may include blue or white, and this can be painful for some people.

Previous studies have shown that applying pressure in the arm can improve blood flow in one of the main blood vessels in the arm. We want to see if this is the case in those with excessive vasoconstriction, and whether applying pressure increases blood flow. If this is the case, this may open the wat for further studies in people who have the established condition of Raynaud's phenomenon and do not respond to current treatments.

Who is organising and funding the research?

This study is not being directly funded, however research into intermittent negative pressure is part of broader research within the Vascular Medicine department, funded by Otivio AS, Oslo, Norway. The study is being organised by Professor Faisel Khan.

Why have I been invited?

You have been invited as you may fit the criteria for the study group. We want to establish whether there is any benefit of applying pressure to the upper limb in those with excessive vasoconstriction.

Do I have to take part?

No. It is up to you to choose and taking part in this study is completely up to you. You can choose to take part or choose not to take part. If you choose to take part you can stop the study at any time. You do not have to provide a reason for not taking part or for stopping the study. If you do decide to take part, but then later decide to withdraw or are advised by the research team to withdraw from the study, we request your permission to keep and use the information already collected. If you do not give permission for us to keep and use this information then it will be removed from the study. This is considered as your withdrawal of

your consent from participation in this study. No further study-related activities will take place.

What will happen to me if I take part?

A member of the research team will make sure that it is suitable for you to take part in this study.

If you are eligible, you will be invited to the Division of Systems Medicine research department of the Medical School at Level 7, Ninewells Hospital to obtain consent from you prior to performing any study assessments. If you are happy to proceed, we will then start to conduct the study assessments.

All study assessments will be performed in the one visit: these assessments look at the blood vessels in the arm and how they function. All of these assessments are completely non-invasive. Other measurements including body mass index (BMI) and skin temperature will also be taken. These are also non-invasive measurements and they will take no more than 1 hour and 30 minutes to complete.

Blood Pressure and Heart Rate: Blood pressure of the arm being studied will be assessed using a blood pressure cuff and electronic monitor. The cuff will be applied to the upper arm being studied, and electronically inflated. It will then slowly deflate, and the electronic monitor will display the recorded blood pressure and heart rate. This should not be painful, but when the cuff is inflated it may be uncomfortable and the arm may become slightly numb. This should reverse once the cuff deflates.

Body Mass Index: Body mass index is calculated using height and weight measurement. Height will be measured using a stadiometer and weight will be measured using a weighing scale. These measurements will then be used to calculate the body mass index

Skin Temperature: The temperature of the skin is measured using a non-contact infra-red device which is held near the skin to establish the temperature.

Laser Imaging: The function of small blood vessels of your arm will be assessed using a laser which can measure the blood flow of the small vessels just below the skin. Two small, circular chambers will be attached to your skin of your forearm using adhesive tape, which will be removed after the test. With the use of a small electric current, a very small amount of two solutions called acetylcholine and sodium nitroprusside will be applied through your skin to these small blood vessels. The laser will then scan that area of skin, in order to measure blood flow as these solutions are delivered. There may be some mild tingling but no pain.

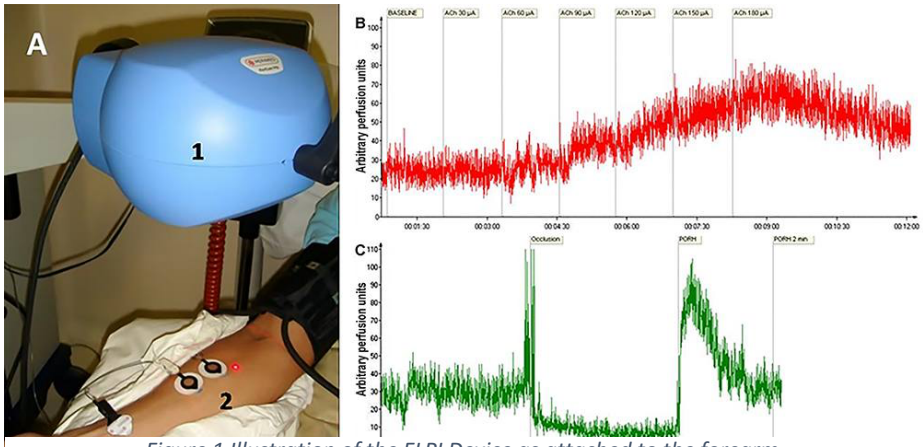

Figure 1 Illustration of the FLPI Device as attached to the forearm

Arterial Stiffness: This is a measure of how elastic or springy your blood vessels are. This will be measured using a specialised blood pressure cuff, called the SphygmoCor which is applied to your arm and thigh. These measures will then be used to calculate a measure of the arterial stiffness.

Figure 2 Photos of the SphygmoCor Device

Flow Ox: The Flow-Ox machine is designed to apply intermittent negative pressure (INP) to your arm, through a pressure chamber shaped as a cylinder, which should sit your arm comfortably. This is connected to a control unit, and the machine will be in use for 1 hour. Operation of the machine will be carried out by the research team.

You will be asked to lie on the bed, which will be at approximately 30°, with both arms resting by your side**.** You will remain in this position for approximately 10 minutes, to allow your circulation to return to resting state. Once acclimatised, blood pressure and heart rate will be measured, then laser imaging and arterial compliance will be measured: these are the 'before' measurements. One arm will then be placed into the Flow-ox tube whilst the other arm remains outside the tube. The arm will be sealed using a rubber seal. Once turned on, the machine will begin to apply pressure, and you should remain and as still as possible for the duration.

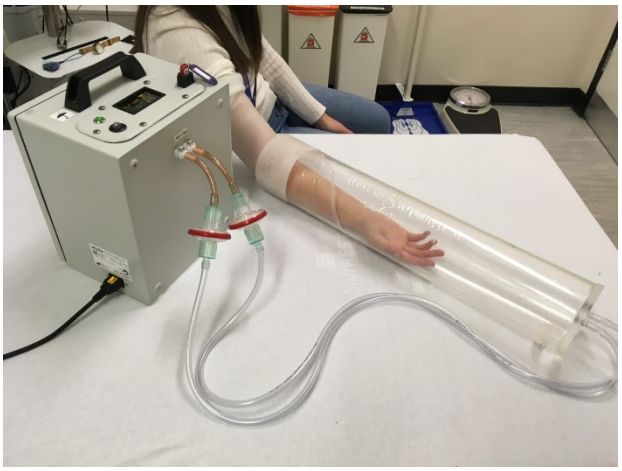

Figure 3 Photo of the Flow-Ox Arm Device

Pressure will be applied in a cycle of 10 seconds of negative pressure and 7 seconds of no pressure. This will continue for an hour. There is a display on the control unit which displays the time of INP application, and after 1 hour, a member of the research team will turn off the machine. Laser imaging and arterial compliance will then be measured again: these are the 'after' measurements.

Once all measurements have been taken, there will be no further requirements or assessments.

Expenses and payments

There will be no cost to you for participating in this study. Reimbursements can be made for reasonable travel expenses. Please discuss this with a member of the research team who can provide you with further details.

What if relevant new information becomes available?

We will tell you about any new information that may affect your participation in the study. For example, changes related to your heart and blood vessels (e.g. blood pressure). You can then decide if you want to continue.

What are the possible disadvantages and risks of taking part?

All study assessments are non-invasive, although there may be some mild tingling and discomfort but no pain. There are no other anticipated risks

What are the possible benefits of taking part?

There will be no direct benefits to you from your participation in the study. However, information from this study may be helpful to those with RP in the future.

What happens when the research study stops?

You will not be required to have any further involvement.

What if there is a problem?

If you are concerned about your participation in the study you have the right to discuss your concern with a researcher involved in carrying out the study.

If you have a complaint about your participation in the study first of all you should talk to a researcher involved in the study. You can also make a formal complaint. You can make a complaint to a senior member of the research team or to the Complaints Department for the University of Dundee

What if I change my mind about taking part or continuing in the study?

You would be withdrawn from the study. Any data or samples already collected with your consent would be retained and used in the study, unless you choose to withdraw your data from the study. We would not collect any further data or samples and no other research procedures would be carried out.

Will my taking part in the study be kept confidential?

Identifiable information about you and the information collected about you during the study will be stored by the University of Dundee. Specified members of the research team will have access to this information and it may be shared outside the research team where it is needed to carry out for this study. Your confidential information collected for this study may be used in ethically approved medical research in the future. Any information which identifies you will be removed before it is shared.

Your identifiable information and coded study information will be stored securely on a password-protected database(s) in the University of Dundee. Specified members of the data management team will also have access to your identifiable information to manage your information and maintain the database.

Your information will be kept securely for 15 years after the end of the study. After 15 years it will be destroyed. If you would like to be informed about future studies that you might be interested to participate we will ask you to sign a consent to allow us to hold your contact details.

Information which identifies you will not be published or shared.

All and any identifiable information about you will be removed and fully anonymised before study data is shared with other researchers in the UK/EU/other.

What will happen to the results of the research study?

When the results become available, they will be submitted to the University of Dundee as part of the BMSc research project. The results will also be submitted to the Trustees of the Wolfson Foundation. The results may also be submitted to medical journals, where they will be considered for publication. All participant data in reported results will be fully anonymised.

You will not be identified in any report or publication

Who has reviewed the study?

The University of Dundee Research Ethics Committee reviews all research proposals, and will examine the proposal and note any objections.

Further information and contact details

Feel free to contact the study team if any further information is needed:

Miss Jody McIntosh
PhD Student
Division of Systems Medicine, University of Dundee
Ninewells Hospital and Medical School
Telephone: 01382 385711
Email: jymcintosh@dundee.ac.uk

Professor Faisel Khan
Professor of Cardiovascular Sciences
Division of Systems Medicine, University of Dundee
Ninewells Hospital and Medical School
Telephone: 01382 385331
Email: f.khan@dundee.ac.uk

Thank you for taking the time to read this information and considering to take part in this study.

If you would like more information, or want to ask questions about the study, please contact the study team using the contact details above.

You can contact us Monday-Friday between 09.00-17.00

Healthy Volunteer Information Sheet

Full Study Title: The potential role of intermittent negative pressure (INP) in improving peripheral blood flow in individuals with excessive vasoconstriction

Name of Chief Investigator: Professor Faisel Khan

We would like to invite you to take part in our study, which is part of the PhD research degree of Jody McIntosh in Vascular Medicine, under the supervision of Professor Faisel Khan at the University of Dundee. Before you take part, we invite you to read and understand why our research is being done and what is involved. One of our team will go through the information and can answer any questions you have.

Please take the time to read through this information carefully.

You can talk to other people about the study if you wish and we will do our best to answer your questions. We can also give you any additional information that you would like. You do not have to decide straight away

What is the purpose of the study?

The purpose of this research study is to see if application of pressure to the arm can improve blood flow. We will be looking at those who have those with what we call 'excessive vasoconstriction'.

Excessive vasoconstriction occurs when cold or changes in temperature result in reversible change in colour, usually of the fingers. Colour changes may include blue or white, and this can be painful for some people.

Previous studies have shown that applying pressure in the arm can improve blood flow in one of the main blood vessels in the arm. We want to see if this is the case in those with excessive vasoconstriction, and whether applying pressure increases blood flow. If this is the case, this may open the wat for further studies in people who have the established condition of Raynaud's phenomenon and do not respond to current treatments.

Who is organising and funding the research?

This study is not being directly funded, however research into intermittent negative pressure is part of broader research within the Division of Systems Medicine, funded by Otivio AS, Oslo, Norway. The study is being organised by Professor Faisel Khan.

Why have I been invited?

You have been invited as you may fit the criteria for the study group. For us to establish whether any findings are significant, we also need a group of healthy volunteers to compare against. There will be about 15 healthy volunteers involved in this study.

Do I have to take part?

No. It is up to you to choose and taking part in this study is completely up to you. You can choose to take part or choose not to take part. If you choose to take part you can stop the study at any time. You do not have to provide a reason for not taking part or for stopping the study. If you do decide to take part, but then later decide to withdraw or are advised by the research team to withdraw from the study, we request your permission to keep and use the information already collected. If you do not give permission for us to keep and use this information then it will be removed from the study. This is considered as your withdrawal of

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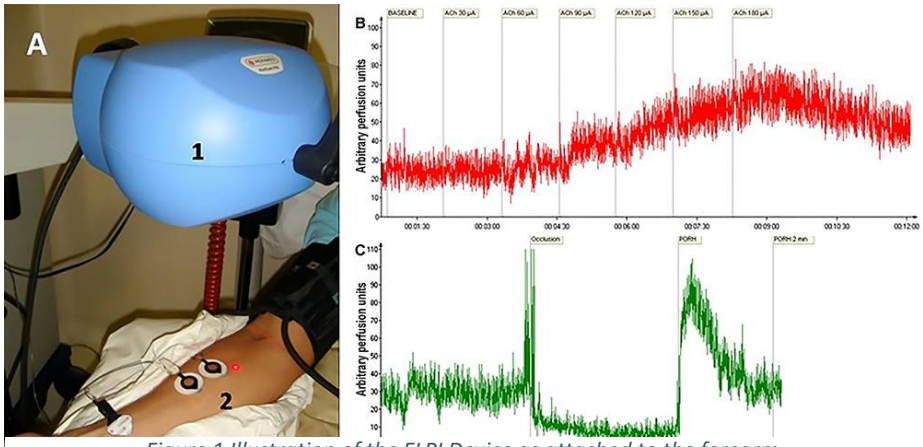

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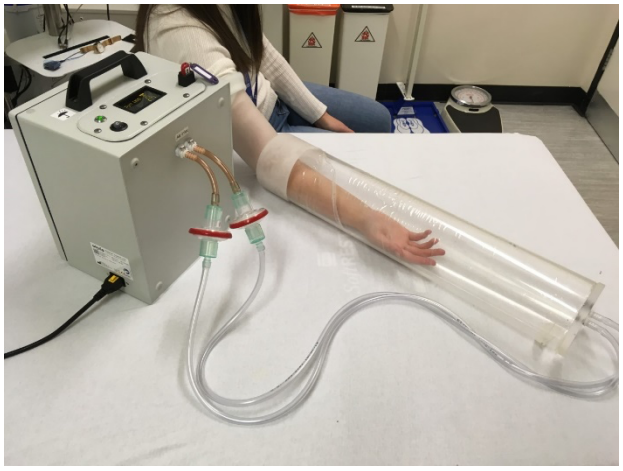

Figure 3 Photo of the Flow-Ox Arm Device

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Expenses and payments

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What if relevant new information becomes available?

We will tell you about any new information that may affect your participation in the study. For example, changes related to your heart and blood vessels (e.g. blood pressure). You can then decide if you want to continue.

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There will be no direct benefits to you from your participation in the study. However, information from this study may be helpful to those with RP in the future.

What happens when the research study stops?

You will not be required to have any further involvement.

What if there is a problem?

If you are concerned about your participation in the study you have the right to discuss your concern with a researcher involved in carrying out the study.

If you have a complaint about your participation in the study first of all you should talk to a researcher involved in the study. You can also make a formal complaint. You can make a complaint to a senior member of the research team or to the Complaints Department for the University of Dundee

What if I change my mind about taking part or continuing in the study?

You would be withdrawn from the study. Any data or samples already collected with your consent would be retained and used in the study, unless you choose to withdraw your data from the study. We would not collect any further data or samples and no other research procedures would be carried out.

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Who has reviewed the study?

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Further information and contact details

Feel free to contact the study team if any further information is needed:

Miss Jody McIntosh
PhD Student
Division of Systems Medicine, University of Dundee
Ninewells Hospital and Medical School
Telephone: 01382 385711
Email: jymcintosh@dundee.ac.uk

Professor Faisel Khan
Professor of Cardiovascular Sciences
Division of Systems Medicine, University of Dundee
Ninewells Hospital and Medical School
Telephone: 01382 385331
Email: f.khan@dundee.ac.uk

Thank you for taking the time to read this information and considering to take part in this study.

If you would like more information, or want to ask questions about the study, please contact the study team using the contact details above.

You can contact us Monday-Friday between 09.00-17.00

Mr Stuart Suttie
Department of Vascular Surgery
Ninewells Hospital and Medical School
University of Dundee
Email: stuartsuttie@nhs.net

Date:

Dear _____

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

We would like to invite you to our research study which is a part of a PhD degree. The purpose of this research study is to see if periods of pressure to the leg can improve blood flow in people who have disease of the blood vessel known as peripheral arterial disease (PAD). Previous studies have shown that periods of pressure can have a beneficial effect in patients with lower limb blood vessel disease. We wish to see whether this application of this pressure by the boot is helpful in slowing down the process of PAD in patients with established conditions. If so, this may offer a novel treatment option and may help reduce the number of leg amputation performed.

This study is looking for people like yourself, who have been diagnosed with PAD. Participation in this study is completely voluntary. Please read the Patient Information Sheet, which will better explain the procedures of the study, and if you decide to take part, please fill and return the reply slip included in this letter to the Chief and Principal Investigators (Professor Faisel Khan and Miss Jody McIntosh, respectively) in the pre-paid envelope. If you have any questions, please do not hesitate to get in touch.

We look forward to hearing from you.

Best Wishes

Mr Stuart Suttie
Consultant Vascular Surgeon

Reply slip:

Study title: Mechanistic Insights into changes in peripheral blood flow following intermittent negative pressure

Chief Investigator: Professor Faisel Khan

Principal Investigator: Miss Jody McIntosh

I am interested in participating in the research project,

I am happy to be contacted by a member of the research team to discuss my involvement.

Name: _____

Signature: _____

Contact Telephone Number: _____

E-mail address: _____

Visual Analog Scale (VAS) for pain measurement

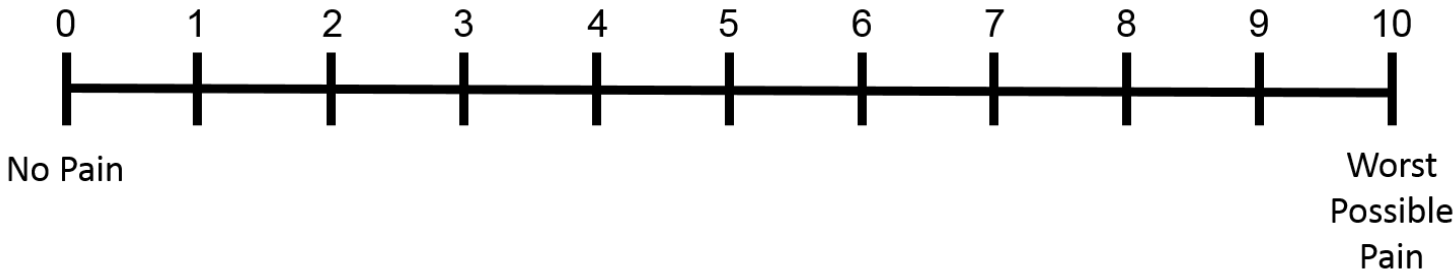
Mechanistic Insights into changes in peripheral blood flow following intermittent negative pressure.

Participant Identification Number:

Date(s):

Baseline

Circle a number from 0 to 10 that best describes your pain with 0 being no pain at all to 10 being the worst possible pain you have ever felt.



Last visit

Circle a number from 0 to 10 that best describes your pain with 0 being no pain at all to 10 being the worst possible pain you have ever felt.