The effects of lower limb intermittent negative pressure on foot circulation and wound healing

- Experimental and prospective studies exploring the acute circulatory and clinical effects of intermittent mild ambient subatmospheric pressure to the lower leg and foot

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PhD Thesis

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PREFACE

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Updates

Since this thesis was approved by the thesis committee, revised versions of Paper IV and V (see p. 6) have been published:

- Sundby ØH, Høiseth LØ, Irgens I, Mathiesen I, Lundgaard E, Haugland, Weedon-Fekjær H, Sundhagen JO, Sanbæk G, Hisdal J. **Intermittent negative pressure applied to the lower limb increases foot macrocirculatory and microcirculatory blood flow pulsatility in people with spinal cord injury.** *Spinal Co*rd 2017. Dec 28. doi: 10.1038/s41393-017-0049-8. [Epub ahead of print]

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List of papers

This thesis is based upon the following original research papers, which are referred to in the text by their Roman numerals:

Paper:

I. Sundby ØH, Høiseth LØ, Mathiesen I, Jørgensen JJ, Weedon-Fekjær H, Hisdal J. **Application of intermittent negative pressure on the lower extremity and its effect on macro- and microcirculation in the foot of healthy volunteers.** *Physiol Rep.* 2016 Sep;4(17). pii: e12911. doi: 10.14814/phy2.12911.

II. Sundby ØH, Høiseth LØ, Mathiesen I, Jørgensen JJ, Sundhagen JO, Hisdal J. **The effects of intermittent negative pressure on the lower extremities' peripheral circulation and wound healing in four patients with lower limb ischemia and hard-to-heal leg ulcers: a case report.** *Physiol Rep.* 2016 Oct;4(20). pii: e12998.

III. Sundby ØH, Høiseth LØ, Mathiesen I, Weedon-Fekjær H, Sundhagen JO, Hisdal J. **The acute effects of lower limb intermittent negative pressure on foot macro- and microcirculation in patients with peripheral arterial disease.** *PLoS ONE* 12(6): e0179001. doi: 10.1371/journal.pone.0179001. eCollection 2017.

IV. Sundby ØH, Høiseth LØ, Irgens I, Mathiesen I, Haugland H, Lundgaard E, Weedon-Fekjær H, Sundhagen JO, Sandbæk G, Hisdal J. **Intermittent negative pressure applied to the lower limb increases foot macro- and microcirculatory flow pulsatility in people with spinal cord injury.** Submitted.

V. Sundby ØH, Irgens I, Høiseth LØ, Mathiesen I, Haugland H, Lundgaard E, Weedon-Fekjær H, Sundhagen JO, Sandbæk G, Hisdal J. **The effects of intermittent negative pressure applied to the lower limb on leg and foot ulcer healing in patients with spinal cord injury: a clinical crossover pilot study.** Submitted.

List of abbreviations

ABPI	Ankle-Brachial Pressure Index
ADP	Arteria Dorsalis Pedis
ATM	Atmospheric Pressure
BMI	Body Mass Index
ECG	Electrocardiogram
HR	Heart Rate
INP	Intermittent Negative Pressure
IPC	Intermittent Pneumatic Compression
kPa	Kilopascal
LDF	Laser Doppler Fluxmetry
MAP	Mean Arterial Pressure
mmHg	Millimeter of Mercury
NPWT	Negative pressure wound therapy
P	Pressure
PAD	Peripheral Arterial Disease
PO_2	Partial Oxygen Pressure
PVR	Pulse Volume Recording
PWAT	Photographic Wound Assessment Tool
RCT	Randomized Controlled Trial
SCI	Spinal Cord Injury
SWC	Standard Wound Care
$TcPO_2$	Transcutaneous Oxygen Pressure
TPR	Total Peripheral Resistance
WSA	Wound Surface Area

Synopsis

Introduction: Lower limb ulceration occurs in about 1 % of the adult population. The numbers are expected to rise sharply in the coming decades due to an aging population and to increased incidence of diabetes and obesity. Patients with late stage peripheral arterial disease (PAD) frequently develop skin breakdown, which may progress into chronic ulcers. Despite advances in medical technology, treatment of PAD and chronic ulcers represents a particularly challenging clinical problem with few efficient non-invasive treatment modalities. The project's overall aim was therefore to explore the acute circulatory and clinical effects of applying ambient intermittent negative pressure (-40 mmHg) to the lower leg and foot.

Design, sample and methods: Paper I: In an experimental study in healthy volunteers (n=23), we examined the acute effects of different patterns of -40 mmHg applied to the lower limb with beat-to-beat analysis on foot circulation (ultrasound Doppler and laser Doppler) and central hemodynamics (heart rate and mean arterial pressure). Paper II: This case study on patients with severe PAD (n=4) and chronic lower limb ulcers explored the effects of 8 weeks of intermittent negative pressure (INP, alternating 10 s of -40 mmHg and 7 s of atmospheric pressure) on ulcer healing. Papers III and IV: We examined the acute effects of INP on foot circulation in patients with PAD (n=20) and people with spinal cord injuries (SCI) (n=24), respectively. Further, we also investigated the changes in blood flow pulsatility during INP compared to baseline (no pressure). Paper V: In a pilot crossover study, we explored the effects of INP + standard wound care (SWC) compared to SWC alone on ulcer healing in patients with SCI and chronic leg and foot ulcers (n=9). In a separate experiment, we examined changes in blood flow in six PAD patients' superficial femoral arteries with stents during application of INP, compared to INP cycles with no pressure (n=6).

Results: Our beat-to-beat analyses on healthy volunteers (Paper I), people with SCI (paper IV) and patients with PAD (Paper III) demonstrated an abrupt and significant increase in foot macro- and microcirculation the first 2-4 s after onset of INP compared to baseline flow (atmospheric pressure), without clinically significant changes in central hemodynamics. While short oscillating INP induced increased foot blood flow in healthy volunteers, constant negative pressure decreased foot circulation (Paper I). In PAD patients (Paper III) and in people with SCI (Paper IV), INP induced increased arterial blood flow pulsatility compared to the 5-min baseline (atmospheric pressure). The case study on four patients with severe PAD (Paper II) showed improved ulcer healing and foot perfusion in all patients. These findings suggest that

INP facilitates ulcer healing and increases foot circulation during and after eight weeks of INP treatment. In the pilot study on SCI patients with chronic ulcers (Paper V), 7 of 9 patients adhered to 90 % of the prescribed INP-protocol and completed the study without side effects. Further, ulcer healing occurred in both treatment groups, and we observed more ulcer healing in the INP+SWC treatment group than those allocated to SWC alone. However, the group difference was not statistically significant in this small pilot study. WSA improvement was seen in 4/4 patients for INP+SWC vs. 3/5 patients for SWC alone, while PWAT improvement was seen in 4/4 patients for INP+SWC vs. 2/5 patients for SWC alone. Our pilot study on SCI patients with chronic lower limb ulcers indicate that INP+SWC may have an additive positive effect on ulcer healing compared to SWC alone. Further, the pilot study showed that INP is feasible as a home-based treatment and that patients were able to adhere to a two-hour daily INP treatment protocol. INP increased blood flow in the stents in the six PAD patients by 44-96 % during negative pressure compared to atmospheric pressure within the INP-cycles.

Conclusions: INP applied to the lower limb increases blood flow pulsatility in foot macro- and microcirculation. This increase in blood flow pulsatility was observed without significant changes in central hemodynamics. The clinical studies on PAD and SCI patients with chronic leg and foot ulcers indicate that INP may have an additive effect on ulcer healing compared to SWC alone. INP may potentially be used to improve tissue perfusion and ulcer healing. Future studies should investigate the potential clinical effects of INP on tissue perfusion and wound healing in adequately powered randomized clinical trials.

1 BACKGROUND

1.1 The Cardiovascular System

The English physiologist William Harvey (1578–1657) is considered to the father of modern experimental circulatory physiology [1]. Harvey performed a series of experiments in the 17th century and described the cardiovascular system with function of the heart as a pump in his work entitled *Exercitatio anatomica de motu cordis et sanguinis in animalibus* [eng: on the movement of the heart and blood in animals] published in 1628 [2]. The physiologist continues to discover and advance the field by integrating the previous concepts established by the early discoveries.

The cardiovascular system is a closed organ system designed to supply needed oxygen, metabolic fuels, hormones, and nutrients to every cell of the body, while also removing waste products and heat generated from cellular metabolism. The cardiovascular (or circulatory) system is organized into two circulatory pathways: the pulmonary circuit, which pumps blood from the right ventricle and through the lungs before entering back into the left atrium and the left ventricle; and the systemic circuit, which pumps blood from the left ventricle through all organs and tissues to provide nutrients and oxygenated blood to the body's cells. After releasing oxygen and nutrients, the blood flows back to the right side of the heart, the right atrium [3].

The heart's job of delivering steady blood flow to the body is vital. The workings of the heart are remarkable, beating on average about 80 beats/min or 4800 beats/hour—or more than three billion heartbeats during a lifetime in a human being 80 years old. The heart muscle is about the size of a fist and is located in the middle of the chest. The degree of circulation is adjusted in accordance with the individual needs of the cell [3]. The rapid blood flow in the tissues is produced by pressure gradient created by the pumping cardiac muscle. The pumping action of the heart generates pulsatile blood flow. This pulsatile flow is further conducted into the arteries and across capillaries, where nutrients and oxygen are delivered to the tissues, and eventually back via the venous system to the heart.

As we breathe, oxygen is transported by convection down the lungs to the terminal bronchioles, due to expansion of lung volume, and then enters the alveoli by diffusion. By the time the inhaled air mixture reaches the lungs, the air has been warmed and humidified by the

respiratory system. Alveolar partial oxygen pressure falls as it is saturated with water vapor and mixed, due to the gas exchange between the alveoli air and the pulmonary capillaries. In the alveoli, oxygen is transported across the alveolar basement membrane and the capillary endothelium by diffusion. From the capillary endothelium, oxygen then goes into the bloodstream, where it binds to the oxygen-binding protein-molecule, hemoglobin. Oxygen-bound hemoglobin travels with the blood down the alveolar capillaries to the pulmonary vein and then to the left side of the heart. The organization of the cardiovascular system ensures that blood is able to pass from the systemic deoxygenated veins to the systemic oxygenated arteries only by first being pumped through the pulmonary system. This organization ensures that blood returning from the body's organs and tissues via the systemic veins in the pulmonary system is oxygenated before it is pumped back to the cells.

Macrocirculation comprises the arteries and veins visible to the eye, which offer little resistance to blood flow. This allows oxygen delivery via the blood to occur over large distances by bulk flow (i.e. movement of the blood due to the pressure gradient). The arteries are thick-walled tubular structures which originate from the aorta (left ventricle) or the pulmonary trunk (right ventricle). Veins have one-way (check) valves which consist of two cusps that fold up. These valves combined with muscle pumps assist veins in returning the blood back to the heart. As blood moves in the direction of the heart, it drives the cusps open, similar to a pair of one-way swinging doors. If gravity pulls the blood backward or if blood begins to back up in a vein, the cusps are instantly closed by the force of the blood itself. This prevents blood from flowing backward [3].

Microcirculation (arterioles, capillaries and venules) is responsible for oxygen delivery to the tissues. The goal of the cardiovascular system is to ensure adequate delivery of blood to the capillary bed in the body's tissues. The ability to maintain an organ's homeostasis and aerobic metabolism depends on the capability to sufficiently and efficiently deliver blood flow with oxygen and nutrients through the microvasculature, and then via oxygen diffusion and substrate transport on to the cell for consumption and utilization. Almost all cells in the body are in close proximity to a capillary, and thus have their own blood supply (exceptions include cells in the cornea of the eye), ensuring the vital supply of oxygen and nutrients and extraction of waste products and carbon dioxide produced by every cell [3].

1.2 Flow, pressure and resistance in the cardiovascular system

Hemodynamics is the study of the relationship among physical laws that govern the flow of blood through vessels in the body [3]. The driving force (driving pressure) that propels blood through the body and through a tissue or a vessel is the pressure difference between two points (i.e. the proximal and the distal part of the vessel). In the cardiovascular system, blood flows from the left ventricle (high pressure) to the right atrium (low pressure). According to Ohm's law, current (I) equals the voltage gradient (ΔV) divided by the resistance (R), $I = \Delta V/R$ [4]. Applying the equivalent of Ohm's law (known as Darcy's law in hydraulics) to hemodynamics, the volume flow rate (Q) is determined by the pressure gradient (ΔP) and the resistance (R) to flow:

$$Q = \frac{\Delta P}{R} \quad [1]$$

The pumping motion of the heart produces (dynamic) pressure potential, which drives the fluid flow. The pressure potential drops throughout the cardiovascular system due to resistance. A pressure gradient or perfusion pressure, ΔP = inlet pressure (P_1) – outlet pressure (P_2) (e.g. the difference between arterial and venous pressure) is necessary for flow to occur. An increase in driving pressure could be accomplished by an increase in the volume, frequency, filling and heart's force of contraction (i.e. increased effect of the pump). The average pressure over time in the arteries is the mean arterial pressure (MAP). MAP drives the blood through the vasculature to serve the tissues during the cardiac cycle [4].

The movement of blood has a given resistance. Resistance measures how difficult it is to move blood through a vessel. Resistance impedes blood flow. Resistance is caused by friction between the blood and the vessels, and internal friction in the blood (viscous forces). In principle, resistance may depend strongly on the flow rate and flow regime (e.g. friction).

The factors determining the resistance (for laminar flow in a perfect cylinder), are described in the Hagen-Poiseuille's equations:

$$R = \frac{8 \times \eta \times L}{\pi \times r^4} \quad [2]$$

R is the resistance to flow (mmHg x time/volume)

$\pi/8$ is the mathematical constant, and are related to the circular cross-section of the tube

L is the length of the vessel or pipe

η is the viscosity of the blood

r is the radius

Combing the two above equations [1 and 2], we get Hagen-Poiseuille's equation [3]. Hagen-Poiseuille's equation [5] states that volume flow rate (Q) of Newtonian fluids through a rigid tube is inversely proportional to tube length (L) and fluid viscosity (η) and is proportional to the pressure gradient (i.e. the pressure drop) across the tube (ΔP) and the tube radius (r) to the fourth power [5]:

$$Q = \frac{\Delta P \times \pi \times r^4}{8 \times \eta \times L} \quad [3]$$

where

Q is the volumetric flow rate (volume/time)

ΔP is the pressure difference (mmHg)

From equation [1] and [3] it is obvious that flow depends on a pressure difference, and that even small changes in vessel radius would have a very large effect on resistance to flow (inversely proportional to the radius to the 4th power according to equation [3]). According to Hagen-Poiseuille's equation, a reduction in vessel radius would increase the resistance to the fourth power of the change in radius (increases its resistance to flow approximately 16-fold). Further, if a blood vessel's diameter were double, the flow rate would increase 16 fold. Consequently, a 50 % reduction in the radius would result in an increase in resistance by

a factor of 16. Hydrodynamically, this would cause flow to decrease by a factor of 16. The strong dependence on vessel radius should also hold for real blood vessels, even if equation [3] does not apply. On the other hand, the vessel length does not change significantly, and the viscosity of blood is usually kept within a relatively small range. Blood viscosity may change during certain conditions, however. For example, for each one degree Celsius decrease in temperature, blood viscosity increases by approximately 2 % [6]. Blood viscosity also increases as blood flow velocity decreases. Conversely, increases in hematocrit levels (for example during dehydration) are accompanied by a corresponding increase in blood viscosity[6]. Increased blood viscosity reduces blood flow, and consequently, higher blood pressure is required to achieve tissue perfusion.

In the cardiovascular system, the local regulation of flow in blood vessels is regulated by vasodilation and vasoconstriction, and as stated in the above equation [3], only small changes in the radius of the vessel is needed to bring about large changes in flow. This estimation by Hagen-Poiseuille's law is, however, based on the following assumptions: (i) the laminar flow conditions prevail; flow is steady, nonturbulent and nonpulsatile, (ii) blood behave as a Newtonian fluid; viscosity is independent of shear forces (flow rate), pressure and temperature ; (iii) the vessels are long, nonbranching, rigid, straight (without any irregularities) and cylindrical tubes [4]. While water and many fluids may follow Newtonian behavior, blood consists of large proteins and large amounts of red blood cells with unique mechanical behavior. Condition (iii) clearly does not hold for real blood vessels. Hagen-Poiseuille's equation assumes, for simplicity, that the flow is laminar through a cylindrical pipe of constant cross section and that there is no acceleration of fluid in the pipe. Blood is however a non-Newtonian fluid. For non-laminar fluid or turbulent flow, this leads to larger pressure drops than what Hagen–Poiseuille's equation considers. Also, the cardiovascular system does not consist of rigid and straight tubes. The cardiovascular system does not meet these strict characteristics. Regardless of these differences, the Hagen–Poiseuille's fluid law demonstrate the main influence of a blood vessel's radius on resistance and flow. Thus, the formula is useful for approximation for explaining how blood flow changes under normal physiological conditions and how pathological change of the vasculature and blood viscosity influence blood flow and pressure. The equation is used in this thesis to give a reasonable explanation of the suggested effect of flow when applying subatmospheric pressure.

The formula in equation [1], when applied to the whole cardiovascular system, could be replaced by: CO = MAP / TPR, where Q = flow output of the heart or cardiac output (CO), P = mean arterial pressure (MAP), R = total resistance of all vessels in the cardiovascular system or peripheral resistance (TPR). Accordingly, MAP will increase if total peripheral resistance (TPR), cardiac output (CO) or both increase, or MAP will decreases if TPR, CO or both decrease. Further, provided that MAP is kept constant, the equation also states that blood flow increases if TPR decreases and vice versa.

Since flow depends upon the pressure gradient and peripheral resistance, therapeutic modalities to enhance arterial inflow are targeted towards altering these factors, e.g. vascular surgery, vasodilator drugs, reactive hyperemia therapy, intermittent pneumatic compression therapy etc. The ability for blood arteries and veins to passively change their diameter is an important function of the vascular system and is discussed below.

1.3 Energy for blood flow - potential and kinetic energy

Blood flow has velocity and mass, and therefore, kinetic energy. When blood pumped by the heart exerts pressure towards the vessel walls, the pressure extending the vessel walls represents potential energy. Potential energy is the ability to do work [7]. Kinetic energy is the result of fluid flow [7]. The driving force of for flow is not always the difference of pressure (e.g. the delta P between the arterial and venous side of the circulatory system), but the difference in total fluid energy between two points [3]. The general statement that blood (or fluid) flow from higher to lower pressure is somewhat inaccurate. The true driving force is the difference fluid energy.

Total fluid energy (E_{total}) in the circulatory system is the sum of following three relations: Pressure energy + Gravitational energy + Kinetic energy. Thus, $E_{total} = P + \rho gh + \frac{1}{2}\rho v^2$, where potential energy is due to the pressure field generated by the heart and the vascular elasticity; the gravitational potential energy is rho (ρ) or blood mass density (1060kg/m^3) * gravitational acceleration on the surface of the earth (g) * height of the fluid column (h); and kinetic energy is ρ * velocity squared (v^2) / 2 (ρv^2 / 2). The pressure gradient adjusted for gravity is considered the driving force for blood flow. In practice, however, conservation energy determines how blood flow changes between two points [3].

Bernoulli's equation states that fluid flows steadily from one point to another downstream; and that the sum of potential energy (from lateral pressure) and kinetic energy (from fluid velocity) flowing through a tube remains the same (i.e. energy is conserved; Bernoulli's equation has to be adjusted to account for friction), provided that flow is uniform and continuous, and there is no frictional loss (heat transfer). Bernoulli's equation = $P_1 + ½\rho v_1^2 + \rho g h_1 = P_2 + ½\rho v_2^2 + \rho g h_2$, where P_1 is pressure at point 1 and P_2 is pressure at point 2; $½\rho v_1^2$ is kinetic energy at point 1; $\rho g h_1$ potential energy at point 1; $½\rho v_2^2$ is kinetic energy at point 2; and $\rho g h_2$ is potential energy at point 2. Since kinetic energy is proportional to mass multiplied by velocity squared, kinetic energy in the vasculature increases as velocity increases. If flow velocity increases in a vessel (and total energy remains constant), pressure energy will drop. Since flow equals the product of mean velocity (V) times cross-sectional area of the vessel (A) ($Q = V * A$), and diameter is directly proportional to radius squared, $A = \pi * r^2$, a 50 % decrease in diameter in the stenotic region results in a four-fold increase in flow velocity. Since kinetic energy $KE \propto V^2$, kinetic energy increases 16-fold [8].

This has implications for pathophysiology, such as when blood flows through atherosclerotic, narrowed regions of a vessel in patients with peripheral arterial disease. In patients with arteriosclerotic stenotic lesions (narrowed region in Figure 1), blood flow velocity increases when stenosis diameter decreases (Bernoulli's equation). Increased flow velocity in the narrowed region (stenosis) results from the transition from potential energy to kinetic energy. If a stenosis occurs in vessels with compliant walls, the reduced transmural pressure (when velocity is high, pressure is low) may result in further narrowing of the stenotic region. Kinetic energy decreases towards pre-stenosis levels in the post-stenosis region, since there is a corresponding increase in diameter in the post-stenosis region (Figure 1). Potential energy increases since kinetic energy is transformed to potential energy (Figure 1). Simultaneously, resistance forces increase in the stenosis region due to increased turbulence caused by the stenotic lesion (and increased friction against the walls). Due to resistance, (friction, turbulence and other means of dissipation), pressure decreases and will only partially recover in the post-stenosis region (Figure 1) [8]. Therefore, fluid energy falls over the length of the vessel (Figure 1). This will potentially cause blood flow to drop in the part of the circulation system affected by the lesion. Turbulence has consequences for the development of peripheral vascular disease, as it may result in endothelial dysfunction and risk of thrombosis formation [9, 10].

In some cases, autoregulation and recruitment of vessels (collateralization) may reduce resistance in the distal vasculature of patients with stenotic lesions. This may result in maintained blood flow at rest. To detect flow in such cases, an ankle-brachial index measurement should be performed after an exercise bout (period with active hyperemia), when distal vasodilation increases blood flow across the stenotic region, further magnifying the pressure drop across the stenosis [8].

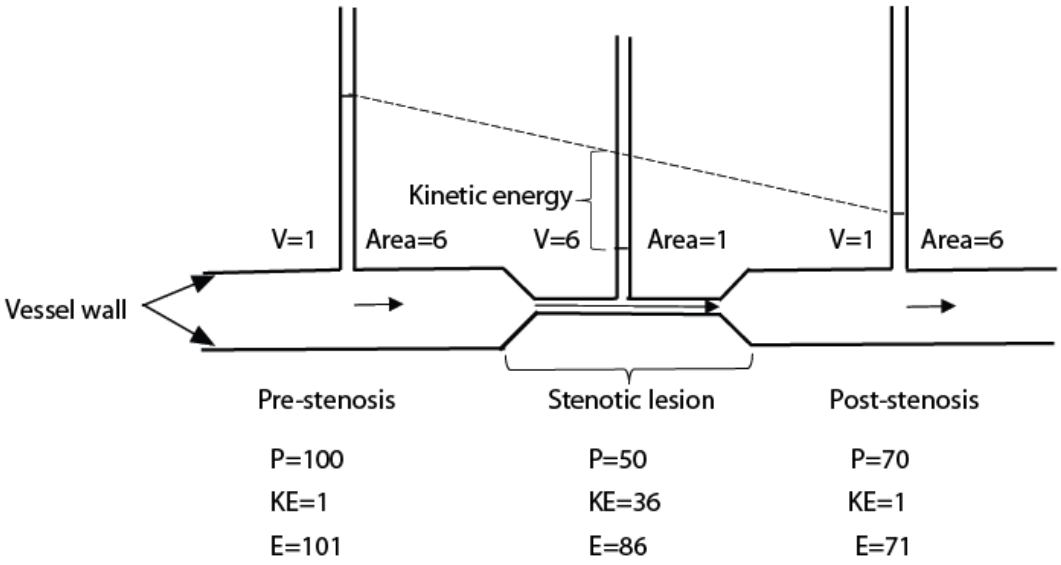

Figure 1. Bernoulli's principle applied to blood flow through stenotic lesion.

The figure model shows constant flow in a tube, illustrating a narrowed (stenotic) region in the middle of the tube cross-sectional area. The figure shows how flow occurs down a gradient of total fluid pressure (E), whereas it flows against the pressure gradient (P) in the stenotic to the post-stenotic area. Thus, fluid flows down a gradient of total fluid pressure (energy), not pressure (energy) gradient. Fluid flows from low to a high kinetic region at a narrowed region. This results in a substantial pressure drop. Bernoulli's equation states that fluid flow through the stenotic lesion of the vessel, potential energy is converted to kinetic energy (flow velocity increases), and potential energy is reduced. Post-stenosis, kinetic energy goes back to pre-stenosis values, since post-stenosis diameter is the same as pre-stenosis diameter and flow is conserved. The ideal fluid circumstances for Bernoulli's equation are not applicable to real fluid flow (arterial blood flow) in human circulation due to energy loss from the transformation of kinetic energy to heat. Therefore, total fluid energy decreases and is therefore not conserved post-stenosis. This is due to increased resistance of the stenosis and likelihood of turbulence. Because of the increased resistance and the turbulence, both the potential energy and total fluid energy fall post-stenosis. Qualitatively the picture above still illustrates the main principle. Figure adapted from Burton 1975 [3].

1.4 Transmural pressure, compliance and recoil pressure

Blood vessels typically consist of three layers: an inner layer (the tunica intimia), a medial layer (the tunica media), and an outer layer (the tunica adventitia). Intima consist of endothelial cells separated from the media by a basal lamina and in larger vessels also connective tissue. The media comprises smooth muscles and components such as elastin, glycoproteins and collagen. The adventitia is comprised of collagen, nerves, blood vessels, and fibroblasts [4]. The vessels' dispensability allow the blood vessels to expand and contract passively with changes in pressure [6].

Transmural pressure (Lat.: trans = across; murus = wall) is the pressure across a wall of a hollow structure [6], i.e. a vessel [inside (intraluminal) minus outside (extraluminal) pressure]; Transmural pressure is the distending pressure, and the increase in transmural pressure increases the diameter of the blood vessels [6]. The ability of a blood vessel wall to expand and constrict passively with alterations in pressure is an important function of the blood vessels. It is one factor controlling vessel radius, and therefore, vasculature flow resistance [4]. The change in volume for a given change in transmural pressure is called the compliance (C) [6]. The equation can be written as change in volume (delta V) divided by the change in pressure (delta P):

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

The ability of a blood vessel to expand and increase in volume with increasing transmural pressure is determined by vessel compliance [6]. The veins are ~20 times more compliant than the arteries [6]. Figure 2 displays the volume-pressure relationship (compliance) for an artery and vein. This illustrates how the slope of the compliance curve is steep at lower pressures for the veins compared to the arteries, indicating that the veins are able to accommodate high blood volumes with only slight changes in pressure. In contrast, the slope of compliance curve is similar to the arteries at higher pressures and volumes [4]. Compliance decreases with vasoconstriction caused by contraction of the vessel's smooth muscles. The ability to regulate vessel tone is an important regulatory mechanism within the cardiovascular system to control arterial blood volume and systemic blood pressure. Stiffening of the arteries caused by aging and arteriosclerosis are other factors which reduces compliance of an artery [6].

An increase in transmural pressure dilates the blood vessel, while the elastic nature of the vessel prevents it from bursting. This equation shows that adding volume to a vascular segment will yield a large increase in pressure inside the vessel if the volume is large and compliance low. The connective tissues and muscles within the wall of the vessel oppose the forces exerted by the increased transmural pressure, which would tend to rip the wall apart. The tendency of the vessel walls to collapse is known as the recoil pressure. When transmural pressure is higher than recoil pressure, as occurs when blood flow in a vessel or air is blown into a balloon, the vessel or balloon expands. Thus, the pressure of dilation or constriction for a vessel segment is the net result of recoil and transmural pressure.

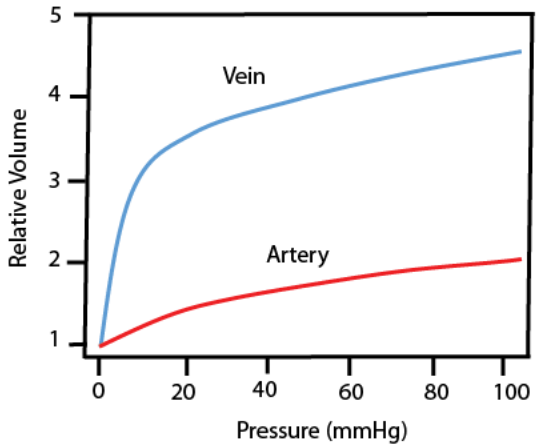

Figure 2. The typical characteristic volume-pressure relationship for large arteries and veins are displayed with their respective lines.

Adapted from Klabunde, Cardiovascular Physiology Concepts [11].

1.5 Arteries of the lower limb

Figure 3 shows a schematic presentation of the arteries serving the lower limb, with the segments used to differentiate the sections of the arteries. The lower limb's main artery is the femoral artery, which is an extension of the external iliac artery, and a terminal branch of the abdominal aorta [12]. In addition to the common femoral artery, the obturator artery branches from the internal iliac artery. This artery, in turn, branches to arteries that supply the pelvic region. The external iliac artery passes posterior to the inguinal ligament and enters the femoral triangle, where it becomes the common femoral artery. In the femoral triangle, the

common femoral artery branches to become the profound femoral artery and then converts to the superficial femoral artery, which descends anteromedially on the thigh in the femoral triangle. The superficial femoral artery is the main artery of the lower extremity, and therefore, essential for the supply of blood to the leg and foot. The superficial femoral artery passes the anterior surface of the thigh via a tunnel known as the adductor canal. The artery supply the anterior thigh muscles during its descent [12]. In the adductor canal it then changes name to the popliteal artery when it passes through the adductor magnus in the distal third of the thigh. As the popliteal artery passes posterior down the thigh it supplies the posterior muscles of the thigh and lower leg, while also giving genicular branches to supply the knee joint. The anterior and posterior tibial arteries commence as the bifurcation of the popliteal artery. The anterior and posterior tibial arteries supply the structures in the anterior and posterior compartments, respectively, of the lower leg. The anterior tibial artery descends on the interosseous membrane, crosses the tibia's lower part at the ankle joint, and changes name to the dorsal pedis artery midway between the malleoli. The posterior tibial artery runs inferiorly accompanied by the tibial nerve entering the sole of the foot via the tarsal tunnel. Perforating branches of the posterior tibial artery also supply muscles in the lateral compartment of the lower leg together with the peroneal artery (see further down) [12].

The arteries that supply the foot are the dorsal pedis, posterior tibial and peroneal (fibular) arteries [12] (Figure 3). In addition, there is collateral circulation between the arteries [13]. Thus, isolated disease of the individual tibial arteries cannot be identified definitively. The peroneal artery originates in most cases from the bifurcation of the tibial-fibular trunk and occasionally from the popliteal artery directly. The peroneal artery supplies the lateral compartment of the lower leg, including the nutrient artery to the fibula [12]. The anterior tibial artery supplies the anterior ankle, and the continuation, the dorsalis pedis, supplies the dorsum of the foot. The posterior tibial artery gives branches to distinct portions of the plantar foot [13].

The main path of arterial blood flow from the aorta to the feet runs serially through the aortoiliac (inflow) area, which constitutes the common iliac and the external iliac arteries. From the inflow area, blood flows to the femoropliteal (conduit) area, which is defined as the superficial femoral arteries and the popliteal arteries. Finally, the blood reaches the tibial (outflow) area, which encompasses the tibial arteries below the knee (leg and foot). Due to the continuous pathway of blood flow, tissue perfusion depends on collateral circulation when a total occlusion is present, i.e. in PAD (Figure 3) [14].

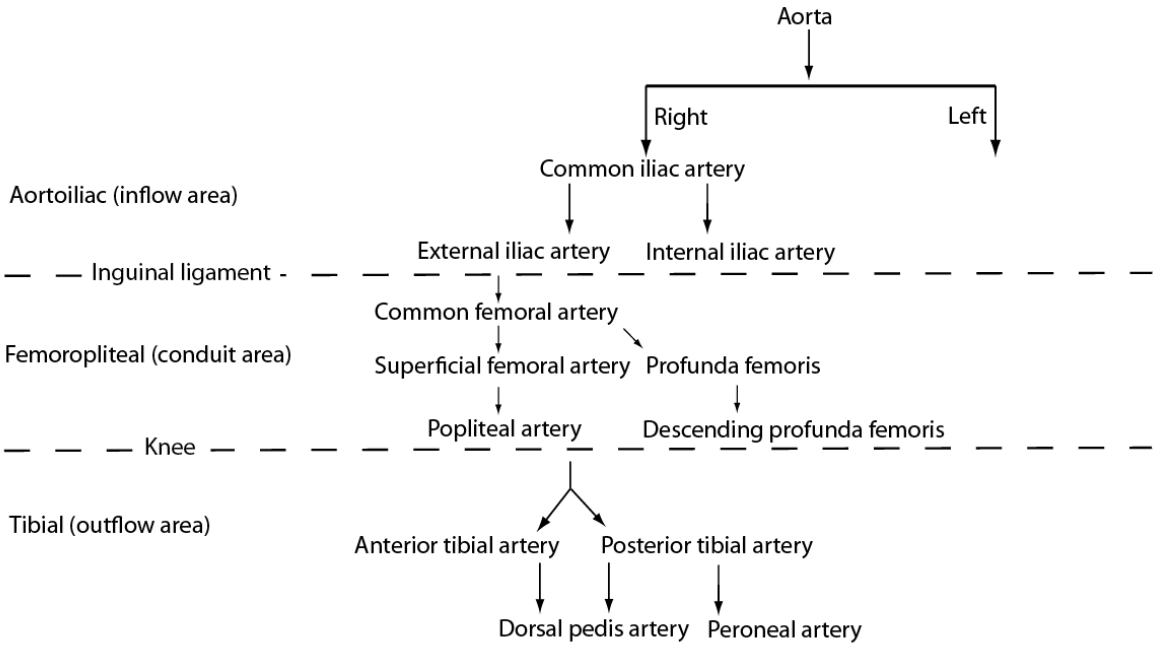

Figure 3. Simple overview of the arteries serving the lower extremity.

Only the right pathway is displayed. Blood flow to the distal leg and foot flows in a continuous path. Thus, when occlusion occurs along this path, circulation to the tissues will depend on collateral blood circulation.

1.6 Blood flow pulsatility

Although few—if any—parameters in healthy physiology are "static", clinical cardiovascular physiology is mostly based on "steady-flow" hemodynamics using physiological variables such as mean pressure and mean flow, or mean cardiac output over time. The observation of pressure contours and flow waves at rest in an individual demonstrate natural flow pulsatility in human circulation.

Pulsatile stretch and shear stress are the two primary forces exerted by flow in the blood vessel [15]. The heart produces pulses when the left ventricle contracts during systole. These contractions generate the needed pressure gradient for flow to occur, and cause a volume of blood to be rapidly ejected into the low resistance aorta and arteries. Compared to the aorta and arteries, higher resistance in the arterioles and capillaries causes the blood to have slower outflow to the arterioles [3]. Therefore, the elastic arteries' dispensability allows them to expand to accommodate the extra blood volume. During the relaxation phase of the heart (diastole), the elastic recoil of the arteries forces blood out into the arterioles. Thus, when the heart ejects during systole, aortic pressure builds up and decays exponentially during diastole. Therefore, the arteries' elastic properties help to transform the pulsatile flow of blood from the heart into a more continuous flow through the rest of the circulation [16]. This storage of energy or elastic reservoir in the arteries and runoff effect which evens out pressure and flow through the vasculature is termed the Windkessel effect [17].

Local hemodynamic (pulsatile) forces have been shown to be important factors for healthy vasculature structure and function and may therefore provide release of paracrine substances from the endothelium and additional beneficial adaptations in the endothelium [18-20]. The shear forces exerted on the endothelium by the flow of blood is analogous to what one feels by rubbing the palms of the hands together. The shear stresses exerted on the endothelium increases correspondingly with increases in blood flow velocity. Such shear stress is thought to be important for endothelial health and repair and a link in generating growth of new blood vessels in the skeletal muscles [21]. Atherosclerosis is more prone to occur in areas where shear stress and blood flow velocity is low [22].

Pulsatility occurs when blood moves through the blood vessels with variable velocity, and the term "flow pulsatility" is therefore defined in this thesis as periodic or rhythmic fluctuations, due to changes in blood flow velocity through a vessel. By contrast, in "steady state" (non-

pulsatile) blood flow, blood moves through the blood vessels at a constant velocity. The importance of pulsatility on tissue perfusion as an unnecessary or necessary physiological phenomenon is still controversial [23, 24]. However, in recent years, several investigations suggest that flow pulsatility may have an important regulatory role in the cardiovascular system to induce increased tissue perfusion and oxygenation [25], and in optimizing tissue perfusion in mathematical models [26] and in humans during extracorporeal circulation using cardiopulmonary bypass [23, 27, 28]. Due to the nature of blood flow in the human body, flow pulsatility may potentially more closely mimic how blood flow through the human vasculature. Although the clinical effects of mode of perfusion (pulsatile vs. non-pulsatile) are poorly understood, research has shown that pulsatile flow generates more significantly hemodynamic energy levels compared to non-pulsatile flow at the same mean arterial pressures and pump flow rates [29]. The extra energy levels generated by flow pulsatility may better preserve regional and global blood perfusion in organs [30-33]. The clinical effects of pulsatile vs. nonpulstile flow is still debated, and others have found no benefits between the two perfusion modes [34-36].

1.7 Subatmospheric pressure

Pressure equals force divided by the area to which it is applied. In Papers I-IV, the term "vacuum" (chamber) has been used synonymously with "negative" and "subatmospheric pressure" to describe pressure below atmospheric pressure. Previously, the term "suction" has also been used in the literature to describe the same phenomenon, i.e. an apparatus that applies pressures below atmospheric pressure [37, 38]. The accurate definition of "vacuum" is a space with zero pressure or a space devoid of all gases, including air [39]. This is of course a situation that has not been achieved with the pressure chambers used in the present project. Such high negative pressures would likely cause tissue damage and would require sophisticated and extremely powerful vacuum pumps. According to the Ideal Gas Law, PV = nRT (where P = pressure, V = Volume, n = number of moles of gas, R = constant, and T = temperature),[40] pressure increases with temperature and decreases with volume. At a given constant temperature, increasing the pressure around a closed environment enclosing a vessel would lead to decreased volume in that specific vessel. In contrast, reduced pressure in the same environment would increase the volume of the vessel. Moreover, in absolute terms, there is no such thing as negative absolute pressure in daily life (since pressure has to be a positive quantity). We have not applied absolute negative pressure, but use the term negative (gauge) pressure with respect to local ambient atmospheric pressure (1 atm = 760 mmHg). Gauge pressure (zero) is relative to atmospheric pressure. In the present thesis, negative pressure and subatmospheric pressure are used as any amount of pressure below zero on the pressure gauge.

1.8 Peripheral arterial disease

1.8.1 Definition

Peripheral arterial disease (PAD) is an atherosclerotic (or thromboembolic disease) causing occlusion or stenosis due to plaque build-up and narrowing of the arteries, exclusive of the coronary and intracranial vessels [41]. These arterial blood flow obstructions result in insufficient oxygen perfusion to muscles and cutaneous tissue [42]. The vasculature in the lower limbs is — in contrast to that of upper limbs — more susceptible to atherosclerotic lesions [43, 44]. Other arteries that are most frequently affected by PAD, besides the arteries of the lower extremities, are the aorta and its visceral arterial branches [45]. In general, therefore, the term PAD refers to chronic atherosclerotic narrowing or blocking of the arteries in the lower extremities. In the lower extremities, PAD affects three major arterial segments: i) the aortoiliac, ii) the femoralpopliteal, and iii) the infrapopliteal (primarily tibial) arteries [45] (Figure 3). PAD is diagnosed by its clinical signs and a specific vascular examination, which may be confirmed by Doppler ultrasound to identify blocked or narrowed arteries [42].

1.8.2 Symptoms and classification

Insufficient blood perfusion to the lower limbs causes dysfunction and pain comparable to the symptom angina pectoris (chest pain) in coronary artery disease [46]. The clogged arteries in the lower extremities restrict blood flow to the leg muscles, leading to excruciating pain, cramping, or aching in the calves, thighs, or buttocks that appears during exercise and is relieved by rest [42]. This symptom of exercise-induced muscle pain that is relieved by rest is called intermittent claudication [42]. In addition to claudication pain and ischemic rest pain, PAD patients may also experience ischemic ulcerations, repeated hospitalizations, revascularizations, and limb loss [46, 47]. Reduced tissue oxygen may not only lead to pain, but also increase susceptibility to infection [48-50], thereby reducing wound healing capacity [51]. Exercise impairment reduces PAD patients' ability to carry out activities critical for daily living [52, 53], leading to negative chronic adaptations in the muscles (and indirectly, in the cardiovascular system), as well as fatigue and muscle weakness [54, 55]. PAD impairments are associated with a marked reduction in quality of life [53, 56] and higher depression rates [57]. Interestingly, even individuals with PAD who are asymptomatic (no claudication pain) are prone to have poorer functional performance, reduced quality of life, smaller calf muscle area, and greater calf muscle fat than an age-matched group of individuals without PAD [58].

PAD is a broad spectrum disease. It includes individuals who are asymptomatic (prevalence: 20-40 % in Western countries) and those with symptoms during ambulation that are relieved by rest (intermittent claudication) (prevalence: 10-35 %). Although 70-80 % of those with intermittent claudication remain stable over a 10-year period, those with intermittent claudication have approximately 2.5 times higher mortality rates than non-claudication patients [42]. The systemic atherosclerosis results in increased risk of cardiovascular events in these patients, and the difference in mortality rates between intermittent claudication patients and age-matched controls has been shown to be relatively unchanged after adjustment of risk factors (smoking, hypertension, hyperlipidemia) [42]. Including asymptomatic and those with intermittent claudication, studies shows that the annual overall major cardiovascular event rate including ischemic stroke, myocardial infarction and general vascular health is about 5-7 % [42]. Further, the risk of angina is two- to three times than for age-matched controls, excluding patients with the most severe stages of PAD [42]. Excluding the most severe stages of PAD, time-courses of 5, 10 and 15 years, all causes morbidity and mortality rates are 30 %, 50 %, and 70 %, respectively [42]. The most severe stage of PAD is critical limb ischemia (prevalence: 1-3 %), involving pain at rest and tissue loss with either ischemic skin ulcers, or — at worst — gangrene [42]. Critical limb ischemia occurs by definition when symptoms have lasted for at least two weeks [42]. Patients with critical limb ischemia experience ischemic rest pain and are at risk of tissue loss, such as leg and foot ulcers, and eventually, gangrene [42]. Patients face major functional limitations and limb threats [42]. Patients with critical limb ischemia have high rates of limb loss and death within a year, and they therefore require aggressive treatment of the disease and of lower extremity ulcers to improve arterial inflow [42, 59]. At the stage of critical limb ischemia, the prognosis is poor, with 6-month amputation rates ranging from 10-40 % [42, 60], and high treatment costs [61]. Approximately 20% of patients with critical limb ischemia will have died 6 months after the onset of critical limb ischemia [42].

ABPI measures offer a quantitative approach to assess the degree of PAD [46]. However, as discussed in the methods section (section 3.1.2), ABPI has its limitations and does not give the full picture of the disease. There are therefore two commonly used classification systems to grade the severity of ischemia and clinical symptoms. Table 1 depicts PAD classification using the Fontaine stage and Rutherford classification schemes, respectively. In these classification systems, asymptomatic conditions are mapped as Fontaine stage I and Rutherford category 0. Intermittent claudication is mapped as Fontaine stage IIa and IIb and

Rutherford stages 1, 2, and 3. The most severe stage, critical limb ischemia is mapped as Fontaine stage stages 3 and 4; Rutherford 4, 5, and 6.

Table 1. The Fontaine stages and Rutherford's classification systems for PAD**.**

Fontaine classification		**Rutherford classification**		
Stage	**Clinical**	**Grade**	**Category**	**Clinical**
I	Asymptomatic	0	**0**	Asymptomatic
II	Mild claudication	I	**1**	Mild claudication
IIa	Mild claudication. Claudication at a distance >200m	I	**2**	Moderate claudication
IIb	Moderate to severe claudication. Claudication at a distance <	I	**3**	Severe claudication
		II	**4**	Ischemic rest pain
III	Ischemic rest pain, nocturnal pain	III	**5**	Ischemic ulceration/minor tissue loss
IV	Ulceration or gangrene of the limb	III	**6**	Severe ischemic ulcers or frank gangrene

Modified table from Fontaine et al. [62] and Inter-Society Consensus for the Management of Peripheral Arterial Disease (PAD), TransAtlantic Working Group (TASC II) [42].

1.8.3 Epidemiology and risk factors

PAD become a global disease in the 21st century, affecting approximately 202 million people worldwide in 2010 [63]. Data on PAD from 2000 to 2010 show and increase in PAD prevalence of 29 % in low and middle income countries, and of 13 % in high income countries [63].

The prevalence of PAD is higher for people with diabetes and smokers, and it increases dramatically with age [64]. In the 1999-2000 National Health and Nutrition Examination Survey of 2,174 participants aged ≥40 years, using ABPI <0.90 as a definition criteria for PAD, the prevalence of PAD was found to be 4.3 % (95 % CI 3.1 % to 5.5 %). That amounted to about 5 million individuals in the United States at the time [65]. Among the different age cohorts, the study recorded PAD prevalence rates of 0.9% for individuals aged 40 to 49, 2.5 % for ages 50 to 59 years, 4.7 % for ages 60 to 69 years, and 14.5 % for all aged ≥70 years [65]. PAD (asymptomatic and symptomatic) is currently estimated to affect 6 % of individuals between the ages of 50 and 60 years, and ~10-20 % of individuals above 70 years of age [42, 63]. In Europe, the estimated prevalence is 40.5 million [63]. A Swedish study on an age-standardized randomly selected sample of 5,080 men and women between age 60 and 90, using questionnaires and ABPIs, found prevalence rates of 18 % for asymptomatic PAD, 11% for intermittent claudication, and ~1 % for critical limb ischemia [66]. The overall prevalence among women has been shown to be higher than among men when using ABPI as a diagnostic criterion alone (12.6 % vs 9.4 % (P = 0.03), and for critical limb ischemia (1.5 vs. 0.8 %, *P* = 0.008) [66]. In the same study, the prevalence of PAD was 7.9 % among those

between 60 to 65 years of age, and 47.2 % among those 85 to 90 years of age [66]. It is important to note that a relatively small proportion (<10 to 15 % over ≥5 years) of patients with intermittent claudication will progress to the most severe stage of PAD, critical limb ischemia [67].

The incidence of PAD is reported less often than its prevalence in the literature [68]. A study from the Netherlands on incidence rates for asymptomatic and symptomatic PAD (n=2327) in men and women used ABPI and the claudication Rose questionnaire [69]. The incidence rates for overall symptomatic PAD for men were 1.7 per 1000 for age 40 to 54; 1.5 per 1000 for age 55 to 64; and 17.8 per 1000 for age ≥65 years. This yields a total incidence rate of 8.2 per 1000 men. Annual incidence rates for women were: 5.9 per 1000 for age 40 to 54; 9.1 per 1000 for age 55 to 64; and 22.9 per 1000 for age ≥65 years, resulting in a total incidence rate of 14.2 per 1000 women. In the literature, there is no conclusive evidence of sex differences [68]. In the Framingham cohort study on 5,209 subjects [70], annual incidence of intermittent claudication (all ages combined) was 7.1 per 1000 in men versus 3.6 per 1000 in women. Based on many studies, more severe PAD, as diagnosed by lower ABPIs or symptomatic disease, tends to be more common in men [68]. In general, there is little evidence on the prevalence and incidence of critical limb ischemia because the condition is less common (partly due to high mortality rates).

PAD is a strong predictor of cardiovascular and cerebrovascular event rates (stroke, myocardial infarction, and cardiovascular death) [42]. Compared to individuals without PAD, the 10-year risk of mortality due to coronary heart disease or cardiovascular disease has been found to be 10 to 15 times greater [71]. The risk of cardiovascular events individuals with PAD is related to the severity of the disease in the lower extremities; the lower the ABPI the higher the risk of cardiovascular event and death [72, 73]. Annually, about 5-7 % of ischemic cardiovascular and cerebrovascular events can be attributed to the progression of PAD [42]. Given that PAD represents systemic atherosclerotic disease that is similar to coronary (atherosclerotic) disease, PAD and coronary disease shares similar risk factors [42]: Known non-modifiable and modifiable risk factors associated with PAD comprises advanced age, race, male gender, family history, smoking, diabetes, hypertension, hyperlipidemia, chronic renal insufficiency, hyperhomocysteinemia, and elevated fibrinogen and C-reactive protein levels [42, 65, 74].

1.8.4 Current treatment strategies for PAD

1.8.4.1 Non-interventional treatments

There is currently no cure for PAD. Treatment strategies focus on impeding PAD progression and providing symptom relief, as well as increasing blood flow through non-interventional and interventional strategies [42, 67, 75]. According to the best evidence available (Class I), clinicians should follow guideline-directed medical therapy (GDMT) (optimal medical therapy) in the treatment of PAD [67]. The treatment goal depends upon PAD severity [42]. In general, for individuals with both symptomatic and asymptomatic PAD, treatment should reduce the risk of cardiovascular morbidity and mortality [42]. Patients diagnosed with intermittent claudication are also advised to improve functional mobility and to minimize potential progression to more severe stages of PAD [42]. For patients with intermittent claudication supervised exercise for a minimum of 30-45 min three times per week is recommended (class 1, level of Evidence: A), while unsupervised exercise currently does not have such strong evidence (class 3, level of Evidence: B) [46]. Further specific treatment modalities include control of comorbidities and risk factors, pharmacological treatments, percutaneous transluminal angioplasty, and revascularization surgery (see below) [42]. For patients with critical limb ischemia, therapies are also geared towards preventing leg amputation [42, 67]. However, the long-term risks of restenosis and graft failure following transluminal angioplasty imply that caution should be taken (for all stages) when it comes to surgical or endovascular interventions [42].

1.8.4.2 Reducing cardiovascular morbidity and mortality

Guideline-directed medical therapy, include behavioural and lifestyle changes such as smoking cessation, improving diet and daily mobility including participation in regular structured exercise program [42, 76]. Further, these guidelines include pharmaceutical drugs in secondary prevention to reduce risk of cardiovascular morbidity and mortality to manage risk factors (tobacco use, diabetes, low-density lipoprotein levels, and hypertension). This include lipid lowering drugs and statins, antiplatelet agents, and blood pressure drugs [42, 67, 77]. The focus of medical drugs is to treat symptoms, and for secondary prevention, to decrease the risk of life- or limb-threatening cardiovascular complications in patients with high ischemic risk [42, 78].

Patients with PAD experience a reduced quality of life due to the discomfort and symptoms of leg pain, as well as the reduction in exercise capacity. As a result, there is a strong interest in developing medical therapies (exercise therapy and/or pharmacotherapy) to improve these symptoms [42]. Patients with symptomatic PAD (intermittent claudication) are advised to be treated with current recommendations for medical therapies [67]. Many of the recommendations have limited evidence on functional performance, however, despite an abundance of data demonstrating their efficacy in treating cerebrovascular disease and coronary artery disease [77, 79]. These treatments include aspirin [80] and pentoxifylline [81-83] (while cilostazol has showed benefits for functional improvements with some adverse effects) [84]. Moreover, pharmacological interventions with antiplatelet therapy and statins also have limited data on functional improvements [77]. By contrast, there is a strong positive relationship between the amount of physical activity during daily life measured by vertical accelerometer and an increase in functional status among individuals with PAD [85]. Exercise is a class 1, level of evidence A recommendation for the treatment of intermittent claudication in individuals with PAD [46, 77]. A 2014 Cochrane review [86] found that exercise programs were "of significant benefit compared with placebo or usual care in improving walking time and distance in people with leg pain from intermittent claudication who were considered to be fit for exercise intervention." In the same Cochrane review [86], the authors concluded that there was limited evidence for the benefits of exercise compared with antiplatelet therapy, pentoxifylline, vitamin E, iloprost, and pneumatic foot and calf compression, due to a small numbers of trials and participants. A randomized controlled trial on PAD patients with iliac- or femoropopliteal arterial lesions found no significant difference in effectiveness between supervised hospital-based exercise and endovascular revascularization during 12-months follow-up [87]. However, any improvements with endovascular revascularization found were non-significant, and the authors concluded that endovascular revascularization costs more than the generally accepted threshold willingness-to-pay value, which also points to exercise as the most beneficial treatment approach [87].

A 2013 cochrane review [88] concluded that supervised exercise has clinical beneficial effects on pain-free and maximal treadmill walking distance compared to non-supervised programmes. The authors concluded that there still is a paucity of data on the clinical relevance of supervised versus non-supervised exercise training for PAD patients, and suggest that future studies need to evaluate quality of life or other disease-specific functional outcomes such as walking behavior, patient satisfaction, and costs, in addition to the long-

term effects [88]. A Cochrane review shows that exercise therapy has significant benefits compared to placebo or standard care protocols in improving ambulatory capacity in individuals with leg pain from intermittent claudication who were considered to be fit for exercise intervention [86]. In the treatment of individuals with intermittent claudication, emerging evidence suggests that the outcome with regards to walking distance and quality of life after endovascular therapy combined with medical therapy or supervised exercise exceeds that of supervised exercise therapy [89] or endovascular therapy alone [90]. However, according to a RCT, supervised exercise seems to be most cost-effective first-line treatment for intermittent claudication. In terms of cost-effectiveness, supervised exercise combined with percutaneous transluminal angioplasty (PTA) is also superior to PTA alone [91].

1.8.4.3 Interventional treatments

If non-interventional treatment (medical and exercise therapy) does not succeed, the next line of therapy is to restore blood flow (revascularization) through minimal invasive endovascular treatments such as PTA with or without stenting or open surgery [92]. Endovascular intervention for patients with intermittent claudication is considered when exercise and medical therapy fail to improve physical function and quality of life [67, 92]. Revascularization as a treatment options should be evaluated based upon the patient's severity of clinical symptoms, comrobidity, and their response to medical and structured exercise therapy program [67]. Endovascular revascularization is recommended as a treatment option for intermittent claudicantion with life-style limiting symptoms and hemodynamically significant aortoiliac disease (Evidence I, class A), and hemodynamically significant femoropliteal disease (Evidence IIa, class B-R) [67]. However, the evidence for endovascular revascularization in the treatment of isolated infrapopliteal disease is unknown [67]. Endovascular intervention has the advantage of lower risk than open surgery in patients with comorbidities [92]. Endovascular revascularization is recommended for patients with critical limb ischemia and chronic ulcers or grangrene to restore blood flow to the foot (Evidence class I, level B-R) [67]. In patients with intermittent claudication, revascularization strategies will usually rely on endovascular revascularization, with surgical intervention reserved for those in whom arterial anatomy is not favorable for endovascular procedures [46]. Overview of the different surgical interventions for inflow (aortoiliac disease) and outflow disease (infrainguinal disease) can be found elsewhere [46].

Revascularization usually involves redirecting the blood so that it bypasses the blocked area of an artery (bypass surgery) or inserting a small balloon into the narrowed artery via a catheter, and thereafter inflating the balloon at the site of arterial narrowing to widen the artery lumen [42]. The latter method is called balloon angioplasty or percutaneous transluminal angioplasty (PTA). PTA works specifically by enlarging the flowchannel of a blood vessel narrowed by atherosclerotic plaque by fracturing the plaque and stretching the blood vessels. While balloon angioplasty may be a sufficient treatment on its own, irregular fracturing or recoil of the plaque may compromise the results. Patency following PTA is highest for more proximal lesions (common iliac artery) and decreases for lesions located more distally [42]. Additionally to the above established techniques, a less common endovascular technique is called atherectomy, where a catheter with sharp blades is used to remove plaque from the artery wall. A 2014 Cochrane review concludes that there is poor quality evidence to support atherectomy as an alternative approach to angioplasty in maintaining primary patency at any time interval for PAD patients [93]. Revascularization with endovascular techniques in peripheral vascular disease involves percutaneous transluminal balloon angioplasty (PTA), stents, stent-grafts, and plaque debulking procedures such as atherectomy [42]. Stents are a wire mesh "scaffold" that is permanently inserted in the artery to keep the artery open, and can be combined with PTA to treat PAD. Stents are inserted to support the plaque while it heals, in order to improve patency [42].

These revascularization techniques to treat peripheral arteries may further be divided into angioplasty with cryoplasty, ultrasound angioplasy, laser angioplasty, peripheral cutting ballons, drug-coated ballons, thermal angioplasty, and standard angioplasty; and stenting with self-expandable stents, balloon-expandable stents, and drug-eluting stents. Further, atherectomy (minimally invasive endovascular surgery) may be applied with laser, orbital rotational and directional techniques [94, 95]. The differences and comparison of these specific endovascular techniques in treatment of lower extremity PAD is beyond the scope of this thesis.

Revascularization is not always possible for patients with impaired general health or an absence of reconstructible vessels [96]. An alternative treatment option to increase circulation is pharmacological treatments [76]. However, pharmacological treatments with vasoactive drugs are expensive and the effect of many drugs are still inconclusive and debated [67]. Naftidrofuryl

oxalate is the only treatment likely to be considered cost-effective [97]. Moreover, the long-term effects of pharmacological treatments are unclear [97].

Table 2. Overview of current common approaches to increase blood flow in the treatment of PAD.

	Solutions	**Application**	**Main considerations**
Endovascular and surgical revascularization	Stents (various types)	Holds the arteries open	Expensive treatment [98], limited options if occluded area is long. Restensosis is the primary limitation of aortoiliac stenting, but the restenosis rates are lower than after infrainguinal interventions [92]. In-stent restenosis rate in bare metal stents is 25 % to 50 % within 12 to 24 months, depending on the location of implantation [99-101]. In general, for bare-metal stenting of lesions in the superficial femoral artery, there is a short term benefit in primary patency, but no long-term benefit in addition to angioplasty [102].
	Bypass	New channel past occluded area	Frequently used, efficient, with amputation-free survival between 50-75 %, depending on type of bypass [103].
	Percutaneous Transluminal Angioplasty (PTA)	Non-invasive procedure using catheters to block open occlusions	Simple and fast technique with high initial clinical success, but with limited durability, and frequent need for monitoring and repeat procedures [104, 105]. Less cost effective compared to supervised exercise in treatment of intermittent claudication [91]. Produces unacceptable high restenosis rates ranging from 40 % to 60 % at 12 months in complex lesions and often requires re-interventions [106]. Especially high restenosis rates when the complex lesions are in the carotid, renal, femoropopliteal, and tibio-peroneal arteries [42].
	Atherectomy	Removing (debulking) plaque from a blockage	Limited evidence. Not recommended above angioplasty [93]. Complications include embolism and vessel dissection [93].

Pharmacological/Medical therapy	Pharmaceutical therapy (vasoactive drugs): Prostanoids (Prostaglandin and Prostacyclin), Pentoxifylline, Naftidrofuryl Oxalate Cilostazol. Drug treatment - statins and antiplatelet agents in secondary prevention of coronary and cerebrovascular disease in PAD patients - treat comorbid conditions (blood glucose management, hypertension, obesity, smoking cessation)	Patients with intermittent claudication and critical limb ischemia aimed at symptom relief and slowing progression of PAD -	• Conflicting evidence on pharmaceutical therapy's effect and cost-effectiveness compared to endovascular and surgical treatments [107]. • Prostanoids. Prostaglandin E-1 may have a clinical benefit by reducing ulcer size, and current data supports the use of prostanoids in patients unsuitable for lower limb revascularization, or for whom revascularization attempts have failed [74]. In general, limited evidence for their effects in intermittent claudication [108]. A 2010 Cochrane review [109] did not find any conclusive evidence that prostanoids provided long-term benefit. Prostanoids may have efficacy regarding rest-pain relief and ulcer healing [109]. Iloprost (synthetic prostacyclin analogues) is recommended in treatment of severe PAD, Fontaine stages III and IV), in cases where reconstructive vascular surgery or percutaneous transluminal angioplasty is no longer possible[1]. Iloprost may reduce rest pain and ulcer size [74]. However, iloprost is not approved in all European countries. Iloprost may also have favorable results regarding major amputations. The evidence for iloprost in the treatment of saving total amputations compared to placebo is ambiguous [109]. Iloprost costs about €3000 for a 4 week course for the most efficient treatment protocol, requires daily hospital stay for more than 6 hours for 4 weeks. Not recommended in the UK[2]. • Naftidrofuryl oxalate is recommended as a possible treatment for intermittent claudication [3]. • Cilostazol improves pain-free and peak distance in claudicants by up to 40 % compared to placebo [78], with some adverse side effects [110]. • Naftidrofuryl, buflomedil or pentoxifylline show no additional benefit with regard to reduction of amputations and wound healing [74].

[1] National Institute for Health and Care Excellence (2017, July 5). Retrieved from: https://www.nice.org.uk/advice/ESUOM24/ifp/chapter/About-iloprost
[2] National Institute for Health and Care Excellence (2017, July 5). Retrieved from: https://www.nice.org.uk/advice/esuom24/chapter/Key-points-from-the-evidence
[3] National Institute for Health and Care Excellence (2017, July 5). https://www.nice.org.uk/guidance/ta223

Non Invasive	Pneumatic compression devices (i.e. Art-Assist device manufactured by ACI Medical Inc., San Marcos, California) and neuromuscular electrostimulation devices (i.e. Geko™ device, manufactured by Firstkind Ltd, High Wycombe United Kingdom)	Patients with edema, and sometimes limb ischemia diagnosed with severe PAD who are not candidates for revascularization via angioplasty or surgical intervention	The positive compressive force exerted is a hazard for certain patients with fragile skin or with ulcers at the location where pressure is applied. Limited clinical evidence on wound healing, pain management, and limb salvage [111, 112]. Pneumatic compression devices are painful when used for a long time. In general, lack of high quality long-term clinical evidence [111]. Neuromuscular electrostimulation devices exploit the same mechanism as the pneumatic compression technique but instead of using physical pressure to empty veins and thereby facilitate arterial inflow, the system uses nerve stimulation to activate muscle which then empty the veins [113, 114].

	Exercise therapy	Patients with asymptomatic and symptomatic PAD. Physiological benefits: increases maximal oxygen uptake, skeletal muscle oxidative capacity, and endothelial function, while reducing systemic inflammation [115]	Recommended by consensus guidelines for patients with intermittent claudication. Strong basic science rationale and imposes other general health benefits [42], Compliance is reduced with non-supervised exercise. Supervised exercise is costly, but studies indicate that it is more cost-effective than PTA and stenting for claudicants with aortoiliac disease [98, 116]. Remains largely under-utilized treatment modality [117]. Exercise may have low physiological adaptations in some late stage PAD patients [118]. Supervised exercise has shown statistically significant benefit on maximal and pain-free walking performance compared with non-supervised regimens [88]. For claudicants who are considered to be fit for exercise intervention, exercise programs have significant benefits compared to placebo or standard care in improving maximum and pain-free walking distance [86]. An RCT on PAD patients with iliac or femoro-popliteal arterial lesions showed no significant difference in effectiveness between endovascular revascularization alone compared to supervised hospital-based exercise alone during 1-year follow-up [87]. Further, any improvements with endovascular revascularization were non-significant. "Endovascular revascularization costs more than the generally accepted threshold willingness-to-pay value, which favors exercise" [87]. However, increasing evidence now supports the use of a combination of endovascular revascularization and exercise therapy, compared to exercise therapy alone [89, 119].

1.8.5 Limitations to existing PAD treatment strategies

There is a critical gap in treatment options between behavioural change and surgical intervention, which makes PAD progression almost inevitable. Currently, the treatment for PAD starts only in later stages with the development of pain and ulcers. Chronic arterial ulcers often require endovascular or surgical revascularization, through techniques such as transcutaneous angioplasty, insertion of stents and bypass surgery. If these procedures cannot be performed or if they are insufficient, the patient ultimately has to undergo an amputation or receive only palliative care.

For prophylactic treatment of PAD, there are few alternatives other than behavioral change: smoking cessation, dietary improvements, and increasing physical activity. These prophylactic treatments are well-documented, but compliance is dismal. The cost of supervised exercise is also too high to be offered to all patients. Several devices such as compression devices [120, 121] and neuromuscular stimulation devices [122, 123] have been evaluated for treatment of PAD. Most of these systems (if not all) were originally developed to prevent the development of deep venous thrombosis often experienced in elderly patients confined to bed rest for long periods of time after surgery. The pneumatic compression devices work by facilitating venous return through physical compression or activation of the leg muscles, which forces blood back to the heart [120].

In summary, compliance with non-interventional treatment and behavioural recommendations is poor [124-126]. Current non-surgical solutions are not cost-effective and suffer from insufficient clinical data and inferior usability (uncomfortable and long treatment duration) [42]. The most efficient solutions to rapidly increase blood flow—surgical intervention—require either frequent or long visits to health care providers. With recent advances in both medical therapy and invasive therapy, there is an ever greater number of treatments available for symptom relief and disease modification. Nevertheless, current outcomes remain poor, especially in later stages of PAD [59, 127]. There is demand for improved wound care in order to reduce limb loss and amputation rates in the later stages of PAD [59]. Given anticipated increases in PAD worldwide [64], research related to primary and secondary treatment is needed. Research for the PAD population should emphasize: (i) PAD-focused systemic therapies intended to reduce the risk of ischemic events in the target population; and (ii) the continued development of limb-specific therapies in order to increase ulcer healing and reduce amputations rates, particularly for critical limb ischemia [77].

1.9 Chronic leg and foot ulcers

1.9.1 Definition

Skin lesions are divided into acute and chronic wounds or ulcers [128]. The definition depends on the time period since they appeared and/or whether they heal in an adequate manner [128]. This definition does not take into account the ulcer etiology. An ulcer (or wound) is defined as skin breakdown with "full thickness depth" with no re-epithelialization of the center of the ulcer, which may also extend to involve deeper subcutaneous tissue at the level of muscle and bone [129]. Chronic ulcers refers to ulcers that will not heal or that show a slow healing tendency, as well as sores that keep returning [129]. Slow or impaired healing (chronic) is further specified by a time frame of at least 4 weeks [129]. Impaired healing implies that the ulcer has failed to heal in an orderly and timely manner due to interference with the normal healing process. Due to the nature of chronic ulcers, they have also been called "hard-to-heal" ulcers [129]. Although many researchers use the term "leg ulcer" to imply a chronic wound in the lower leg and foot [129, 130], the term "chronic ulcer" is used throughout this thesis to describe chronic wounds.

1.9.2 Etiology

Chronic ulcers develop when there is a mismatch between the cutaneous tissues' metabolic demands and the supply of circulation to provide adequate oxygen and nutrients to the cells. The distorted supply and demand ratio may — if left untreated — increase the risk of developing infection. This may progress into sepsis or gangrene, and thus, become potentially limb-threatening [129]. In general, the slow healing is not simply explained by the ulcer's depth and size, but rather, by an underlying pathogenetic factor that needs to be removed to induce healing [131]. Chronic ulcers are particularly common in the lower leg and foot [129]. According to the most common etiology, ulcers are frequently categorized into mixed ulcers (combined etiology), arterial ulcers, venous ulcers, diabetic/neurothrophic ulcers or ischemic/decubitus ulcers (pressure ulcers) [129].

Wound healing involves complex mechanisms with many factors contributing to the healing process [132]. As noted in the table below, the literature identifies several principal local and systemic factors that can potentially disrupt healing (Table 3).

Table 3. Local and systemic factors affecting wound healing.

Local factors	Systemic factors
Oxygenation	Age and gender
Infection	Stress
Foreign body	Ischemia (Poor circulation leading to inadequate perfusion and oxygenation)
Venous insufficiency and edema	Diseases: diabetes, PAD and immunocompromised conditions (e.g. cancer, radiation therapy)
Repetitive trauma to the wound	Obesity
	Inadequate nutrition

*Adapted from Guo and DiPietro [132].

1.9.3 Epidemiology

Although patients with severe PAD or SCI frequently experience chronic ulcers of the lower leg, there are no reliable estimates of their prevalence and incidence [133, 134]. A rough estimate suggests that chronic ulcers of the lower leg are prevalent in about 1-2 % of the general adult population in industrialized countries [130]. There is an increased incidence of ulceration as a result of the ageing population and increased risk factors for atherosclerotic occlusion, such as smoking, obesity and diabetes [135]. In industrialized countries, the most common type of leg and foot ulcers are venous ulcers, which constitute approximately 45-60 % of all ulcers. Arterial ulcers account for another 10–20 %, diabetes ulcers account for 15-25 %, and 10-15 % have a combination of etiologies [129].

Pressure ulcers are common, especially among the elderly and among bedridden patients. Prevalence is reported to be between 20-43 % in hospital and nursing homes in European studies [136, 137]. Among people with chronic SCI in long-term care, estimated prevalence ranges from 29-40 % [138-141]. Research on older long-term care residents with chronic SCI shows that roughly 50 % of stage II [142] and 95% of stage III and IV pressure ulcers do not heal within eight weeks [143]. Pressure ulcers are one of the most common secondary long-term medical complications (years: 1, 10, 15, 20) following SCI [140].

1.9.4 Economic costs to society

Chronic ulcers are a major health concern, estimated to affect approximately 6.5 million people in the United States annually [144]. In 2006, roughly 500 000 total hospital stays in the US were registered with pressure ulcers as a diagnosis, with a total annual cost of $11 billion

[145]. The true cost of treating chronic ulcers worldwide is unknown. Several studies have estimated the costs, but they only capture costs directly covered by the health system, not the indirect costs of treatment [146]. Annual costs related to the treatment of chronic wounds in the United States amount to an estimated US$25 billion [144]. One study estimated an average cost per patient of US$ 129,248 for hospital treatment (one admission) of stage IV hospital-acquired ulcers and their associated complications [147]. Rough estimates suggest that annual cost in European countries for diabetic foot ulcers are € 4-6 billion, and the cost of leg and foot ulcers counting only venous ulcers to be € 6.5 billion [148].

Individuals suffering from lower limb ischemia and chronic ulcers have high morbidity and mortality, and significantly reduced quality of life [149-151]. Chronic ulcers also burden the health care system, as these hard-to-heal ulcers have high levels of recurrence and are costly to treat [148, 152, 153]. Chronic ulcers are not prevalent in the healthy physiology, but more commonly affect individuals suffering comorbidities such as diabetes, obestity, PAD, and neurological disorders [138, 144, 153]. Despite advances in medical technology, there are no effective noninvasive treatment methods to increase blood flow in patients with poor circulation. Specifically, non-invasive methods to prevent adverse events after revascularization, and to treat and prevent occurrence of chronic ulcers, and limb-threatening ischemia are lacking [154-156].

1.9.5 The wound healing process

Wound healing in general is a complex process and interrelated mechanisms are involved to regenerate new tissues [131]. However, a key factor in cell regeneration and wound healing is supply of adequate oxygen and nutrients to the harmed cells. This requires additional oxygen and energy through the reparative processes, including cell proliferation, infection resistance and defense, angiogenesis, and collagen synthesis [157]. Therefore, regardless of the etiology of an ulcer (being arterial, venous or diabetic in origin), an adequate blood supply is a consistent requirement at all stages of healing.

The healing process may be impaired when blood flow is limited by disease or physical factors, e.g. in patients with PAD and diabetes, where the blood supply to the legs is too low and thus the body is unable to respond to the traumatic challenge. The optimal treatment of foot ulcers in these patients would be to increase blood flow to the leg and skin and soft tissue. Ulceration of the lower leg is a frequent condition in PAD patients due to reduced

microcirculation in the extremities [129]. Reduced blood flow and tissue oxygen may also lead to increased susceptibility to infection and reduced wound healing capability [158, 159]. This imbalance results in a breakdown of the skin and ulceration – a process that, if untreated, will lead to gangrene and loss of the extremity [131]. Unfortunately, leg and foot ulcer treatment is often insufficient [160, 161], causing avoidable complications and unnecessarily extended healing times [162]. In sum, despite the burden chronic wound has on to the individual and society, the conundrum of what is optimal treatment for chronic ulcers continues. There is a lack of research on wound management, and a critical gap in knowledge about what interventions effectively improve microcirculation and wound healing [135].

1.10 Negative pressure as a treatment modality – a historical overview

The hypothesis that air pressure can be manipulated to treat diseases and inadequate peripheral circulation is an old idea. The first record of the use of negative pressure seems to be from Hippocrates' use of "cupping" around 700 BC, China 1000BC [163]. The first description of applying subatmospheric pressure around a limb to treat diseases was described in the mid-19th century [164, 165]. Murray reported the use of subatmospheric pressure (-40 mmHg) for 20 min to treat cholera and various diseases [164, 165]. Later, Victor Junod published a doctoral thesis on the subject in Paris [166]. He introduced Dr. Junod's Hemospasic Apparatus [166-168] to treat a variety of diseases. The apparatus involved placing the limb in a metal boot, sealed with silk or rubber cap, and lowering air pressure (-80mmHg) via a pump. Subatmospheric pressure was maintained for several hours or until the patient fainted [168]. Around the same time, another researcher, W. Reid Clanny, published a paper called "Apparatus for removing the pressure of the atmosphere from the body or limbs" in the treatment of cholera [169].

In 1887, the English researcher Edgar Bluck [170] for the first time introduced a novel method alternating negative (-80 mmHg) and positive pressure (+80 mmHg) to increase circulation in the limbs [171]. In the early 1900s, a German Professor, August Bier, introduced [172] "hyperemic treatment". August Bier applied a "suction" apparatus customized to different body parts to "increase this beneficent inflammatory hyperemia resulting from the fight of the living body against invasion."[173]. A comprehensive manual of Bier's hyperemic treatment was published in 1908 by Drs. Meyer and Schmieden [173]. In this manual, the goal was to induce "artificial hyperemia to increase the quantity of blood in a given diseased part of the body." This may be

one of the first documented reports on the use of subatmospheric pressure (-80 mmHg) therapy on skin ulcers. Meyer's apparatus applied suction chambers with constant subatmospheric pressure (~ -80 mmHg) to the body part six times per day for five minutes, with intervals of three minutes between the applications in order to give the edema and hyperemia an opportunity to disappear. The first account of the use of Bier's techniques on four patients with peripheral vascular disease appears to be by Sinkowitz and Gottlieb in 1917 [174]. In that report, Sinkowitz and Gottlieb reported beneficial effects such as increased foot temperature and skin color (as an indirect measure of increased arterial flow), reduced pain, and improved ulcer healing using constant negative pressure therapy on the foot of patients with thrombo-angiitis obliterans (Buerger's sisease). The Bier's method of hyperemia was also reported to be successful on 19 patients with Raynaud's disease in 1931 by Braeucker [175].

In 1933, Herrmann and Reid proposed the use of an "intermittent negative pressure environment" to treat obliterative vascular diseases, a method they named passive vascular exercise (PAVAEX) [176, 177]. Herrmann and Reid applied alternating negative pressure (-80 mmHg) of 10s and positive pressure (+20-40 mmHg) for 5 s, for a treatment duration of 20 minutes to several hours for severe cases [171]. At the same time, another research group with Landis and Gibbon worked with the same idea [178, 179]. Landis and Gibbon presented a modified version of the method applying alternating pressure of 80 mmHg for 5 s and suction of -120 mmHg for 25s to treat various forms of lower limb arterial disease using rapid change (3s) in pressure [179, 180]. Based on the Poiseuille's law of flow that the amount of fluid flow through a rigid tube depends on the fall of pressure along the tube, Landis and Gibbon [179] demonstrated that alternating negative pressure and positive pressure increased the flow of water by 45 to 63 % in an experiment using a circulatory scheme consisting of rigid tubes. The authors applied -120 mmHg for 25 s and positive pressure of 80 to 100 mmHg for 5 s [179]. In the same study, the authors observed increased skin temperature in healthy subjects and in patients with PAD under controlled conditions [179]. Landis and Hitzrot treated patients with diabetes, claudication and rest pain, as well as patients with Buerger's Disease [180]. Unfortunately, the studies using negative pressure treatment modalities predate the development of more advanced and modern means to measure flow changes when exposing the lower limbs to intermittent "suction regimes." There were no tools to determine the success of the therapeutic measures other than to examine changes in peripheral circulation and skin temperature, or to make clinical observations on skin color and wound vitality.

The application of combined suction to expand the vessels and compression to empty the blood vessels of the lower limbs has been reported to show beneficial effects and is reasonable physiologically sound [171, 178-185]. However, the custom-made "suction devices" of the 1930s also suffered some major drawbacks. First, they required the clinician to operate the devices during each session. Patients could not use the devices on their own. Secondly, the material used to build the devices at the time made them cumbersome to operate, heavy and expensive. Lastly, the air-tight inflexible seal, which was a pneumatic pressure cuff, may have in itself interfered with blood flow.

In 1969, the first report was published using a “Doptone” Ultrasound flow velocity detector to measure the isolated effects of INP (-150 mmHg) on femoral artery flow velocity during alternating pressure of 15 s on and 15 s off [37]. Smyth [37] reported increased subjective walking distance in 30 of 42 claudicants and wound healing on three patients with Raynaud’s disease after six weeks of INP treatment. Additionally, using the plethysmography method to measure arterial inflow in 11 of the patients, the same study also reported a 282 % increase in mean resting flow in the calf after six weeks of INP therapy for half an hour twice a week [37]. In the same study, a reactive hyperemia test after 2-min occlusion on the same 11 patients demonstrated increased peak flow of 300 % after six weeks of INP therapy [37]. As others have pointed out of [38], the study had methodological weaknesses relating to the lack of a control group and the use of self-reported peak walking distance. Also, the Smyth study [37] did not measure microcirculatory blood flow in the patients.

In 1974, Gill and Walder [186] investigated the use of INP (-150 mmHg) four weeks. The authors replicated the INP method used by Smyth [37] subjecting the patients’ limb (eight with lower limb PAD and seven with longstanding Raynaud’s disease of the hands) to 30 min of INP three times per week. In addition, five patients served as control subjects. That study reported increased finger blood flow (venous occlusion plethysmography technique) and arm skin temperature among the seven patients with Raynaud’s disease after four weeks INP treatment. The authors observed no changes in leg skin temperature or cutaneous blood flow of the toes (venous occlusion plethysmography) in those with obliterative arterial disease during INP. Resting and post-exercise calf muscle blood flow (^{133}Xe Clearance technique) increased, in addition to increase in resting ankle systolic pressure increased. The five controls remained unchanged. In those with Raynaud’s disease, significant increase in finger blood flow and arm skin temperature were observed, and these increases were maintained

throughout the four week period. These findings correspond to the aforementioned findings by Smyth [37] who also observed increased resting blood flow and peak hyperemic response after INP therapy [37]. The authors concluded that flow to ischemic limbs can be increased, at least temporarily by means of INP using 150 mmHg subatmospheric pressure [186].

Himmelstrup et al. [187] investigated in the early 1990s the effects of alternating negative and positive pressure oscillations of 5-10 s, with each treatment lasting 15 min, in 22 patients with PAD (stable intermittent claudication) in a two-month randomized placebo controlled cross-over study (Vacusac Intl. aps, Denmark). That study found increased pain-free walking distance, peak walking distance and ABPIs in the intervention groups, while the patients allocated to the placebo groups did not increase their ABPIs [187]. Using a similar protocol as the Himmelstrup et al. study [187], Mehlsen et al. [188] also found increased peak walking distance and increments in the ADP threshold for platelet aggression in the intervention group, with no changes in the control group, in a randomized placebo double-blinded trial on PAD patients. Also, these two latter studies used a different methodology to induce negative pressure pulses. In these studies, the lower limb and body up to the level of the axillae was enclosed within an flexible pressure chamber consisting of inner layer of porous felt and an out layer made of an airtight plastic bag [188]. An electronical pump applied oscillating subatmospheric pressure when air was drawn out of the airtight plastic bag for 5-10s. The device also simultaneously applied a small physical positive pressure to the skin at the point of contact between felt fibers and the surface of the skin during the suction phase [188]. Although the randomized controlled studies by Himmelstrup et al. [187] and Mehlsen et al. [188] indicate increased peripheral circulation and showed beneficial clinical results with robust clinical design, they did not evaluate cutaneous blood flow or use other modalities to quantify the acute effects of INP on macro- and microcirculation.

In a study by Rein et al [189], INP (-40 mmHg) combined with heated water increased core body temperature when applied to part of an upper extremity, compared to conventional forced-air warming. This indicated that the INP may increase circulation by similar INP protocol (alternating 10s on and 7s off) used in the present thesis. However, there was no measure of blood flow in the extremity exposed to INP. In another study, Rein et al. [190] examined the effect of INP on the prevention of hyperthermia. In that study, nine healthy volunteers were exposed to passive ambient continuous heat stress, and randomized to either a commercial device that applied constant negative pressure and cooling temperature of 19°C sealed around the hand

(CoreControl®, Avacore Inc. Ann Arbor, MI) or INP (alternating 10s -40 mmHg and 7 s atmospheric pressure) in combination with a temperature of 24°C that covered the lower and upper arm [190]. Rein et al., showed that INP and cooled water more effectively prevented an increase in core temperature compared to the CoreControl® system [190].

The effect of increased vascular transmural pressure on peripheral circulation by different methods has been extensively studied. These methods include: i) applying subatmospheric pressure to an extremity [191-193]; ii) passively lowering an extremity below horizontal position [194]; and iii) inflating pneumatic cuffs to subdiastolic pressure to induce venous congestion [195]. Skagen and Henriksen [196] demonstrated that applying local constant subatmospheric pressure of -20 mmHg to part of the upper limb results in only a 9 % increase in vascular resistance, and that this caused a 7 % decrease in peripheral blood flow. Furthermore, increasing negative pressure to -40 mmHg resulted in a more pronounced vasoconstriction and 43 % reduction in peripheral blood flow, corresponding to an approximately 90 % increase in vascular resistance. Further increases in local negative pressure beyond -40 mmHg to -150 mmHg caused no additional increase in vascular resistance [196]. These results suggest that the major vasoconstriction occurs when transmural pressure increases from 20 mmHg to 40 mmHg [196]. The vasoconstriction induced by local constant subatmospheric pressure was abolished by a local nervous blockade induced by lidocaine. This indicates that the vasoconstriction is mostly attributable to the local sympathetic venoarteriolar axon reflex mechanism [196]. Based on these findings and the authors' conclusions [197], the results indicate that the dominant part of the vasoconstriction induced by the local axon reflex occurs when vascular transmural pressure increases from 20 to 40 mmHg.

1.9.1 Negative Pressure Wound Therapy

Currently, negative pressure is widely used as a non-invasive treatment modality with the aim of facilitating wound healing and drawing excess fluid from the focal wound area [198]. The method is known as negative pressure wound therapy (NPWT). NPWT refers to wound dressing systems which apply subatmospheric pressure to a local wound area surface. Alternative names are vacuum-assisted closure (V.A.C.®, Kinetic Concepts Inc.(KCI), San Antonio, TX), subatmospheric pressure therapy, topical negative pressure (TNP), and sealed surface wound suction [199, 200]. The NPWT method is widely used as a wound management

treatment modality in health care systems around the world [201]. During NPWT, negative pressure is applied intermittently or continuously to the local wound bed [201, 202]. However, the application of INP during NPWT is completely different from the INP method used in the present project for three reasons. Firstly, the INP method presented in this thesis induces mild negative pressure to a larger area of the lower limb, in contrast to applying subatmospheric pressure only to the focal wound bed. Secondly, the NPWT method uses either "continuous", "intermittent", or "variable" pressure therapy. The use of intermittent pressure is somewhat ambiguous because intermittent negative pressure — in the case of NPWT — is used with alternating cycles of 5 min of constant subatmospheric pressure and 2 min off [202, 203]. Also, NPWT commonly applies higher negative pressure levels (-80 mmHg to -125 mmHg) than the INP method (-40mmHg) used in the present thesis [204, 205]. NPWT applies a subatmospheric pressure to a focal wound area while INP applies the subatmospheric pressure to the whole lower leg and limb and therefore subjects mechanical and suction forces to all the subcutaneous tissues and vasculature of the lower leg and foot. Since the INP -method applied in the present thesis induces mild INP to a larger area of the lower limb, it could theoretically facilitate increased arterial inflow in all six angiosomes (i.e. perfuse all areas of skin and underlying tissues in the lower limb vascularized by the source arteries) of the lower leg and foot due to mechanical dilation of the tissue and vessels exposed to INP. As opposed to placement of an extremity in a subatmospheric pressure chamber, NPWT foam may irritate periwound cutaneous tissue or not adequately seal around fragile skin [206]. Thus, application in patients can be challenging since the contact area may create new pressure points.

The exact working mechanism of NPWT is unknown. However it is believed that NPWT results in several effects [40, 207]. Many interrelated factors are believed to occur, including removal of excess interstitial fluid, increase localized blood flow, increased local vascularity and associated decreased bacterial colonization, and mechanical forces which may induce faster healing [208, 209]. It has been shown that the degree of wound contraction increased gradually with increasing levels of negative pressure during NPWT [210]. Furthermore, blood flow during NPWT has been found to decrease in the wound bed and in close proximity to the wound bed, while blood flow was found to slightly increase 2.5 cm from the wound edge [210]. The decreased tissue perfusion beneath the wound area suggest that NPWT should be used with caution in areas with poor vascularity [211, 212]. The possible reported effects of NPWT on acute and chronic wound healing [208] may, therefore, work through different mechanisms of

action than increased arterial inflow and tissue perfusion. This includes mechanical tissue deformation, prevent infection and drainage of excessive fluid and debris of the wound [213].

According to a 2015 Cochrane review, there is limited evidence available regarding the clinical effectiveness of NPWT in the treatment of chronic lower leg and foot ulcers [201].

1.11 Significance of the present thesis

Patients with chronic leg and foot ulcers, such as those in the late stages of PAD, diabetes, and SCI represent a large and growing group of patients with high morbidity and mortality, enormous cost to the society, and significantly reduced quality of life for the affected individual [206, 214-216]. Despite numerous advances in interventional radiology and vascular surgery, there is a lack of treatment options for patients with chronic ulcers. In total, chronic leg and foot ulcers affect approximately one percent of the adult population in developed countries [217], with a prevalence of 3-5 % in the population over 65 years of age [129]. It is important to develop additional non-invasive treatment options for chronic ulcers as significant comorbidity in PAD and renal failure patients limits the possibility of surgical treatment.

Despite the long history of using negative pressure as a treatment modality, there is a lack of studies investigating in detail the isolated effects of INP applied to an extremity. Moreover, there are currently no reports on the treatment's effectiveness for different patient groups. There are few well-designed studies and studies using different INP methodologies. Moreover, most studies have not examined the isolated effects of INP. As a result, the circulatory effects of INP on the lower leg and foot are not well described in the literature. Primary methodological weaknesses include:

- Relatively small sample sizes
- Different populations
- Poorly defined criteria for patients' stage of disease
- No control group
- No comparison to other treatment modalities' detailed flow measurements
- Differently engineered pressure devices (positive and negative pressure vs negative pressure only)

- Differences in pressure cycle lengths and durations of treatment
- Differences in criteria for patient treatment (stage of disease)
- General lack of consistent methodology to describe flow changes during INP application.

Table 4 in appendix 3 summarizes the key studies discussed in this thesis and in papers I-IV that have applied negative pressure to an extremity or the lower body. Considering the aforementioned limitations of the previous studies, a direct comparison between the studies to this thesis' papers (Papers I-IV) is not feasible.

2 AIM AND OBJECTIVES

The general aim of this dissertation was to explore and elucidate the acute effects of mild intermittent negative pressure (-40mmHg) applied to the lower leg and foot on foot circulation and central hemodynamics in healthy subjects and in patients with lower limb ischemia (PAD) and SCI. We hypothesized that intermittent negative pressure (INP) would induce an increase in foot macro- and microcirculatory blood flow fluctuations (flow pulsatility) in healthy subjects and in ischemic limbs compared to the no pressure baseline. If the initial hypotheses were confirmed, the secondary aim of this project was to explore the potential clinical effects of repetitive INP use on wound healing for chronic lower leg and foot ulcers. In the pilot and feasibility study on SCI patients with chronic leg and foot ulcers, we hypothesized that eight weeks of daily INP therapy plus standard wound care (SWC) would improve ulcer healing compared to SWC alone in SCI patients with chronic leg and foot ulcers.

2.1 Research objectives

1. Elucidate the acute effects of applying different patterns (10 s on – 7 s off-15 s on - 15 s off, 30 s on-30 s off, 2-min on) of mild (-40 mmHg) negative pressure oscillations to a limited part of the lower limb on changes in foot macro- and microcirculation and central hemodynamics in healthy volunteers.

2. Explore and describe the acute effects of applying mild INP (-40 mmHg) to the lower leg and foot flow pulsatility and central hemodynamics in patients with lower extremity PAD, and in people with chronic SCI.

3. Explore the clinical effects of repetitive INP use applied to the lower leg and foot on foot macro- and microcirculation and ulcer healing in patients with PAD and hard-to-heal leg ulcers.

4. Explore the effects of repetitive INP use plus SWC compared to SWC alone on ulcer healing in patients with SCI and chronic lower leg and foot ulcers.

5. Explore the feasibility of applying INP on SCI patients with chronic leg and foot ulcers, and test if the INP method combined with SWC can be delivered in a home setting.

3 MATERIAL AND METHODS

3.1 Ethical considerations

3.1.2 Ethics Committee and Data Inspectorate approvals

The study protocols presented in this thesis were approved by the Regional Committee for Research Ethics of Eastern Norway (REK) (acute measurements ref: 2014/1967/REK Sør-Øst D, and wound healing study ref: 2015/1318/REK Sør-Øst D), and the Institutional Review Board at Oslo University Hospital, Aker. At the screening visits, all attending participants were given (orally and in writing) an extensive study description with particular emphasis on the use of INP. The study participants were informed and reassured that they had an option to withdraw from the study at any time without providing a reason. All participants gave their written informed consent before the studies began. Additional written informed consent was obtained from patients to publish the ulcer photographs in Paper II. The patients included in the project were not prevented from receiving any conventional therapy. All personnel and staff involved in data collection were bound by an oath of confidentiality. All data used for analysis was anonymous and handled according to standard regulations for clinical data. The studies involved in this project were carried out according to *The Declaration of Helsinki*.

3.1.3 Safety precautions for the use of a subatmospheric pressure device

The INP prototype device (FlowOx™, Oslo, Norway) used in the present doctoral project device is CE marked to facilitate clinical testing (Figure 4). Both the subcontractor, Innokas Medical Oy, and the manufacturer, Otivio AS, are ISO 13485 certified. The FlowOx™ device uses high quality components and parts with high functional capacity designed to reduce the risk of malfunction during early stage clinical testing. The FlowOx™ was originally designed to allow the clinician to customize the software by changing some parameters (time, pressure, pressure-cycles). These advanced features were turned off in the clinical studies (Paper II and IV). The INP device (FlowOx™, Otivio, Oslo, Norway) has safety valves that provide double protection against high pressure situations. When enabled, the software allows a maximal pressure of -65mmHg. The software is pre-programmed to deliver -40 mmHg of negative pressure cycles, and cycle lengths alternating 10 s on and 7 s off. If the pressure cycle deviates significantly from the pre-set levels, the electronic hardware system will shut down

the pump. Additional mechanical (safety) valves within the ambient air line opens the air inlet automatically if pressure falls below -100±20 mmHg.

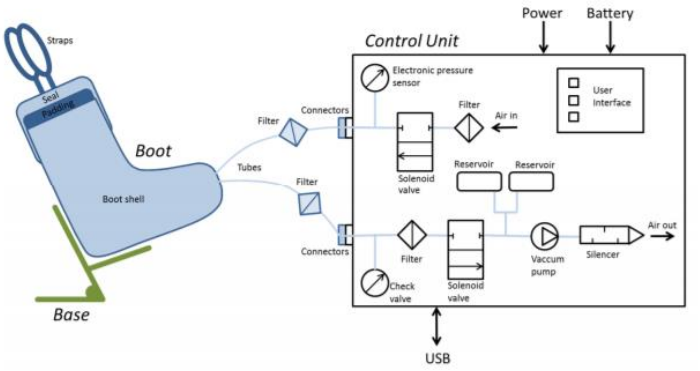

Figure 4. System overview of the FlowOx™ device used in the thesis with its main components.

The subatmospheric pressure chamber (the hard boot shell) is made of rigid medium-density polyethylene plastic designed to be robust enough to withstand forces in excess of those experienced during normal operation and fault conditions.

The elastic seal is made of Thermoplastic Elastomer (TPS-SEBS) coated with a synthetic powder to assist placement. It was purchased from a specialist manufacturer and is CE marked for use with prosthetic limbs and prolonged skin contact.

The hoses which connect the subatmospheric pressure chamber with the control unit (i.e. the INP pump) have been fitted with high efficiency HEPA filters commonly used on anesthesia equipment to prevent infection. These filters protect the pneumatic circuits in the control unit from exposure to potential contaminates from the patients. Each patient in the clinical studies (paper II and IV) were equipped with their own new individual subatmospheric pressure chamber throughout the study.

Data from the hemodynamic examinations were initially recorded on paper. Paper records were kept in a locked archive facility until entered as electronic data into a Microsoft Office Excel database. Finally, the database was anonymized, so that no identifiable data were obtainable other than a unique study ID number.

The use of intermittent negative and positive pressure to the lower extremity has previously been tested extensively [171]. There are also reports of using INP therapy for six weeks with -

150mmHg without any reports of negative events [37]. Also, the change in venous pressure during INP (-40 mmHg) is believed to be less than the pressure changes in the lower extremity during position changes from laying down to standing, or ambulatory activities, and is thus regarded as safe [218]. There were no reports of injuries or any other complications following pressure application in the healthy volunteers or among the patients.

3.2 Study designs

Two different study designs were employed in this project. First, we performed three experimental single-center studies to investigate the acute effects of applying INP to the lower leg and foot on healthy subjects, patients with PAD, and people with SCI. Figure 5 shows the experimental design used in study II-IV. Study II was a case study to explore the potential clinical long-term benefits of repetitive use of the INP-method on wound healing and foot perfusion in patients with PAD and leg and foot ulcers.

Recruited patients were randomized into INP intervention + SWC (A) or SWC alone (B). After eight weeks, patients were re-examined at the vascular laboratory, and then crossed over to alternate therapy, for a total of 16 weeks of study.

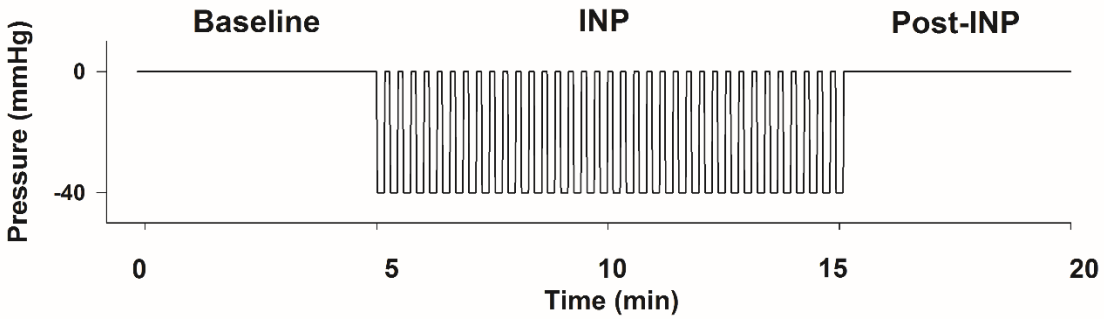

Figure 5. Study protocol used for investigating the effect of foot circulation before, during and after termination of INP in paper II, III and IV.

3.3 Laboratory and study populations

All experiments were carried out at the Section of Vascular Investigations at Oslo University Hospital, Aker from 2014 through 2017. The study populations for papers I-IV consisted of adults >18 years of age. The specific inclusion and exclusion criteria are outlined in papers I-IV.

Healthy participants (Paper I)

The first paper was based on repeated measurements of 23 healthy young volunteers without any known cardiovascular disease, non-smokers, normotensive (<140/90) [age (range) 24 (20-45) years]. The healthy volunteers were recruited from a local University College and from Oslo University. The study included both men and women. Two participants who were included were later excluded from the study due to difficulties in obtaining high quality Doppler signals.

Patients with PAD and chronic leg and foot ulcers (Paper II)

Patients were recruited from the Department of Vascular Surgery at Oslo University Hospital, Aker. Median (range) age was 68.5 years (61-79).

Patients with lower extremity PAD (Paper III)

Symptomatic PAD patients [median (range) age 74 years (63-84)] with Fontaine stage II were recruited from the outpatient clinic at the Section of Vascular Investigations, Oslo University Hospital, Aker. Median pain-free walking distance was 112m (1067-28), and median peak walking distance was 341m (1070-107). The patients had significant stenosis in the aortoiliac, superficial femoral artery, popliteal arteries, or leg arteries and an ankle-brachial pressure index (ABPI) at rest of ≤0.90. Initial vascular examinations were performed by one of the coauthors (JH). Although women have a similar prevalence of PAD compared to men [219-221], an equal ratio of women to men was not feasible in this project for the study on PAD due to the demographic symptomatic prevalence of the disease, with a higher ratio of men to women visiting the outpatient clinic at Aker Hospital.

Patients diagnosed with chronic spinal cord injury without leg and foot ulcers (Paper IV)

Participants with long-term (chronic) SCI [age (range) 57 (29-74) years] were recruited from the weekly exercise training group for people with spinal cord injury at the local hospital, and through the Facebook group for "Landsforeningen for Ryggmargsskadde (LARS)" [eng.: The Norwegian Spinal Cord Injuries Association].

The experimental studies used a convenience sampling process. For the studies on healthy volunteers and spinal cord injuries, we attempted to recruit equal numbers of men and women.

Patients with chronic SCI and chronic lower leg and foot ulcers (Paper V)

In study V, nine patients with SCI and chronic leg and foot ulcers were included [age (range) 57 years (41-74)]. The patients were recruited based upon criteria for chronic ulcers, i.e. ≥6 weeks with leg and foot ulcers without any change in the ulcers during this time. The ulcers were not homogenous and there was a large variety in ulcer size and etiology. A discussion of potential bias and validity of the populations is included under limitations in the Discussion section of this thesis.

One SCI patient withdrew from the intervention group after being assigned to a treatment group (Paper V). During the clinical pilot study on SCI patients (Paper V), patients were closely followed during the study period through the use of telemedicine. The video conference connection was established from Sunnaas Rehabilitation Hospital by collaborating partners consisting of a specialized wound nurse and physician. All communication took place in real time via the Norwegian Health Network and encrypted software. No pictures or audio files were stored during the telemedicine sessions. The work presented in this thesis was conducted in accordance with the Declaration of Helsinki.

The first performed all the experiments (Paper I-IV). None of the participants in the experimental studies withdrew their consent at any time.

3.4 Outcome variables

The outcome variables assessed in this thesis are presented in the following table:

Table 1. Outcome variables in paper I-V.

- Arterial blood flow velocity (cm/s)
    - Change in cumulative up-and-down fluctuations between sequences
    - Changes in blood flow velocity over time (sec by sec)
- Laser Doppler flux (arbitrary units)
    - Change Laser Doppler flux over time (sec by sec)
- Skin temperature (◦C)
- Central hemodynamics [heart rate (beats/min) and systemic blood pressure (mmHg)]
- Ulcer healing
    - Ulcer area (cm^2)

Additional measures in Paper II:

- Trancutaneous oxygen pressure ($TcPO_2$)
    - Changes in mean $TcPO_2$
- Changes in foot circulation over time
    - Changes in skin perfusion pressure (mmHg)
    - Changes in ankle brachial pressure index (mmHg)

3.5 The INP technology and the subatmospheric pressure chamber device

We have in the present thesis used a portable subatmospheric pressure chamber device (FlowOx™, Otivio, Oslo, Norway). The FlowOx™ is a non-invasive Class IIa medical device. Figure 6 shows the custom-made INP device used in the present thesis. The device is designed to increase blood flow to the limbs by applying ambient negative pressure oscillations via an INP generator. The FlowOx™ is developed to be mobile and therefore available for home use. The FlowOx™ device is specifically designed to avoid pressure points. To our knowledge, this device is the only mechanical solution which applies negative pressure to a large area of the lower extremity and which does not exert positive pressure on the limb.

The FlowOx™ technology used in the present project currently involves three main parts:

1. Subatmospheric pressure chamber: A rigid boot with internal padding to prevent pressure points on the leg and skin (disposable and recyclable material). The "single patient use" subatmospheric pressure chamber has been developed through careful ergonomic studies.

2. Control unit: A control box that generates INP. The device can be reused by multiple patients.

3. Seal: A very flexible recyclable sleeve designed to seal the environment between the patient leg and the boot to ensure an airtight chamber. The seal is designed to impose a minimum of pressure.

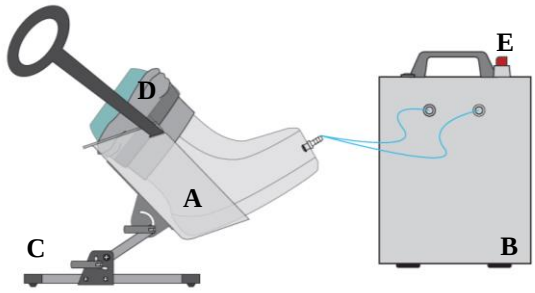

Figure 6. Illustration of the prototype of the custom-made subatmospheric pressure chamber.
The INP unit consists of a subatmospheric pressure chamber (A), a pump with a control unit (B), a base (rack) (C), and a seal (D). The subatmospheric pressure chamber can be placed in the rack (C), which allows optimal adjustments of the height and angle of the leg. The control unit is operated by software with the opportunity to customize the (daily) treatment time as defined by the clinician or researcher. The clinician or researcher can modify the treatment time by accessing a configuration menu held on the USB drive (E) used to operate the unit.

3.6 Measurements of lower limb circulation and ulcer healing

There are various noninvasive vascular testing methods for measuring lower limb macrocirculation. In the present project, participants were examined using Duplex ultrasonography (grayscale and color-Doppler ultrasound), ankle-brachial pressure indices (ABPI), and ankle pulse-volume registration (PVR). Doppler ultrasound was used in experimental studies to examine blood flow velocity in the foot arteries (dorsalis pedis artery or posterior tibial artery).

Microcirculation has been assessed by skin perfusion pressure (SPP) (Paper II) or laser Doppler flux (LDF) (Paper I-IV). Both LDF and $TcPO_2$ were also used in the case study (Paper II) to examine acute changes in foot perfusion when exposing the lower leg and foot to INP in one of the PAD patients with ulcers.

It is important to choose the right instrument in order to ensure measurement validity [222]. Sensitivity and specificity refer to information about the accuracy of the test. Sensitivity is the test's ability to correctly detect the disease (true positive rate) [223]). In contrast, specificity is the test's ability to determine those cases that are truly negative: the percentage of healthy cases correctly identified as not being sick [223]. An ideal diagnostic test has both high sensitivity and specificity [224].

The accuracy of a test is the ability to distinguish sick from healthy cases correctly (proportion of true results: true negative or true positive in a specific population). Accuracy and bias (systematic error) refer to the same concept: the agreement between the reference value (gold standard) and the measured value [225]. Therefore, a greater systematic error would yield a less accurate value [225].

Error is the difference between the reference values and the measured value. Precision refers to the variability (closeness) between repeated independent measurements [226]. High precision therefore indicates consistency in replicating a given measurement around the same value under the same conditions. High accuracy refers to a lack of systematic error [225]. Precision is how repeated measurements show the same consistent results. Precision is threatened by random errors influenced by factors such as the operator, type of equipment used, equipment calibration, environmental factors (humidity, temperature, etc.), and time elapsed between measurements [226]. In order to enhance both accuracy and precision, and reduce systematic bias, experiments and measurements may utilize strategies such as: i) standardizing measurements methods; ii) using experienced and trained operators; iii) refining and adjusting instruments to fit individual body proportions; iv) using automatic and calibrated instruments; v) blinding [225]; and other techniques. Figure 7 summarizes the concepts of precision and accuracy.

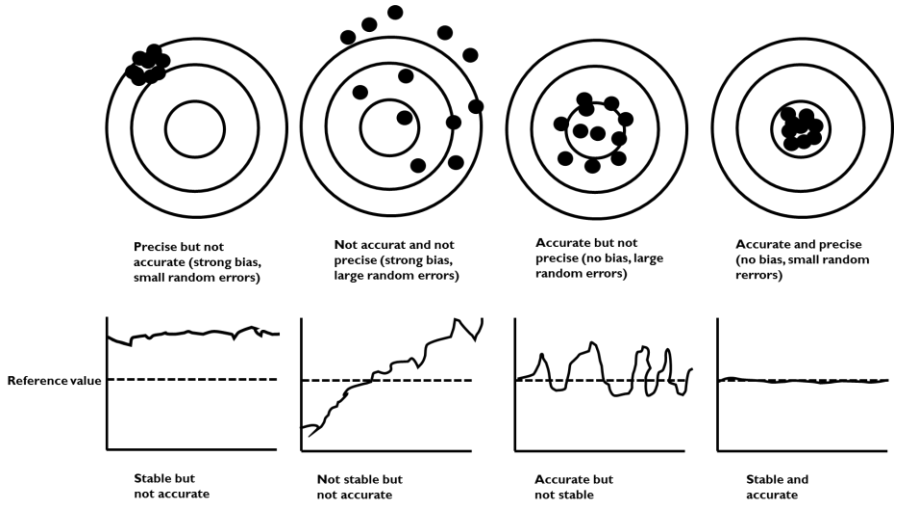

Figure 7. Simple illustration of the terms accuracy and precision.

3.1.1 Doppler ultrasound

Physiology and implementation: Doppler ultrasound measures blood flow velocity. The method is based on changes in the frequency of the reflected ultrasound beam (echo), caused by red blood cells in motion. The transmitted ultrasound beam is backscattered to the transducer. The shift in frequency of the returning echoes compared to the frequency of the transmitted sound waves is called Doppler shift. These measurable changes in frequencies (Doppler shift) are used to calculate blood flow velocity and direction [227].

We used the Doppler effect with a 10 MHz pulsed Doppler probe (SD-50; GE Vingmed Ultrasound, Horten, Norway) to measure blood flow velocity in the dorsalis pedis/posterior tibial arteries [228]. The ultrasound transducer had a fixed angle of about 45° between the sound beam and the underlying skin surface. The instantaneous mean arterial blood flow velocity was calculated by the SD-50 interfaced with the computer for beat-by-beat time averaging, gated by the ECG R-waves. The recorded signal was scanned for maximum amplitude by positioning the probe horizontally and centrally over the artery. Once the optimal signal was obtained, the probe was then fixed in place with several layers of adhesive surgical tape (Micropore Surgical Tape, 3M, MN, USA).

Clinical use and interpretation: Doppler ultrasound is a noninvasive technique used to estimate changes in blood flow by measuring blood flow velocity. Blood flow (often expressed as ml/min) is the volume of blood that is moving per unit of time, while blood flow

velocity is the distance blood travels per unit of time (often expressed as cm/s or m/s). Blood flow in a vessel is related to blood flow velocity as follows: F = V * A, where F = flow, V=mean flow velocity, and A=cross-sectional area of the vessel. The cross-sectional area (A) of a vessel equals pi (π) times the radius squared(r^2), $A = \pi * r^2$.

The relationship between blood flow velocity and blood flow can be expressed as $F \propto V * r^2$ [229]. This equation illustrates that blood flow velocity is proportional to blood flow, provided that the whole cross-sectional area of the artery is illuminated and the vessel radius remains constant. It is important to note that Doppler techniques measure blood flow velocity, and that the only way to know with 100 % certainty that the change in blood flow velocity is actually due to a proportionate change in blood flow is to keep vessel diameter constant. Any vasoconstriction or vasodilation from which the Doppler measurement is performed would in theory affect the blood flow measures. For example, if an intervention causes the lower limb artery to dilate (i.e. due to flow-mediated vasodilation or vasoactive drugs), total blood flow is likely to increase because of the vasculature's in-series organization. The vasodilation would also cause blood flow velocity to decrease (blood flow velocity falls, but blood flow increases). Thus, in this particular context, total blood flow would be underestimated. In contrast, measuring blood flow velocity in a stenotic lesion of an artery would yield a very high blood flow velocity in the stenotic region, even though total blood flow would likely be low.

Triplex ultrasound (color-flow imaging) (10 MHz) transducers), Vivid E9, GE Vingmed Ultrasound, Horten, Norway) were used in the present thesis to screen patients and to verify PAD. An experienced (>10 years) operator (JH), who is also one of the co-authors, performed the Triplex ultrasound measurements. In Triplex ultrasound three modes of evaluations are used simultaneously: Triplex ultrasound B-mode (brightness mode; a grey-scale two-dimensional image of the vessel) and color Doppler (a color scale is used to reflect speed of flow and flow is also colored blue or red to reflect direction of flow) are combined with pulsed-wave Doppler (to show relative velocity and direction), hence the name triplex (Color-flow imaging).

3.1.2 Ankle brachial pressure index

Physiology and implementation: The ankle brachial pressure index (ABPI) is a measure of systolic blood pressure in the arteries supplying lower limbs relative to central, aortic

pressure. ABPI is measured by calculating the ratio of the systolic blood pressure in the ankle (either the dorsalis pedis artery or the posterior tibial artery, whichever is higher) to the systolic blood pressure in the brachial artery [42]. ABPI is a noninvasive and reproducible test [230]. However, to avoid measurement error due to subclavian stenosis, which is more frequent in the left side [231], the higher pressure of the two brachial arteries of either arms is taken when calculating the ABPIs [64]. The ABPI is usually greater than 1.0 in a healthy individual, and an ABPI of <0.9 points to the presence of arterial stenosis within the extremity [42]. The reason that ABPI is greater than 1.0 in healthy individuals is believed to be due to arterial pressure pulse changes from the aorta to the peripheral arteries from pulse wave reflections and wall thickening in the lower extremity arteries. This leads to amplification of the systolic pressure of approximately 10-15mmHg compared to the brachial systolic pressure, while there is a decrease in diastolic pressure [230, 232]. Obstruction of the vessels due to stenotic lesions in major arteries of the lower limbs reduces the systolic ankle pressure relative to the brachial artery systolic pressure [42]. A reduced ABPI in patients with symptomatic PAD confirms that there is a hemodynamically significant occlusion between the heart and the ankle [42].

Clinical use and interpretation: ABPI is considered a first line screening tool in clinical settings and in epidemiological studies in order to objectively distinguish between asymptomatic and symptomatic PAD [64]. The ABPI is used as a differential diagnosis to confirm the presence of PAD, and to provide information about the long-term prognosis [42]. The threshold for diagnosing PAD is commonly set at ≤0.90 at rest, which indicates arterial stenosis in the lower limb [230]. In contrast, elevated ABPIs of >1.40 are indicative of calcified or "stiff" incompressible arteries at the calf, typically found in elderly, end-stage renal disease or diabetic patients [230]. In these circumstances, with elevated ABPI and a suspected PAD diagnosis, a toe brachial index should be measured [67, 230]. An ABPI of <0.50 in nonrevascularized patients with leg ulcers is reported to increase the likelihood of amputation [233].

ABPI has been shown to be highly sensitive and specific in the diagnosis of PAD in patients with significant stenosis [230]. ABPI limitations may include the failure to detect proximal disease in the (infrarenal) aorta and common iliac arteries (inflow disease) in individuals with well-developed collateral circulation and in those with incompressible pedal arteries [234, 235]. In some patients, an exercise test may be warranted to increase ABPI sensitivity [236]. A comparison of ABPIs before and after an exercise bout may reveal PAD, since increased

blood flow across the stenotic lesion will increase the pressure drop across the lesion. This pressure drop results in a fall in peripheral systolic pressure [230]. Accordingly, ABPIs decrease during an exercise bout when there is a proximal stenotic arterial lesion [230].

A review from eight studies on patients age range 35 to 94 years concludes that ABPI is a simple, inexpensive, non-invasive method to identify PAD with severe stenosis [237]. That review found ABPIs to have high specificity (83.3 %-99.0 %) and accuracy (72.1 % - 89.2 %) for an ABPI of ≤ 0.90 in detecting ≥ 50 % stenosis, but there was a wide range in the levels of sensitivities (15 % - 79 %) [237]. Also, sensitivity was found to be low in elderly individuals and patients with diabetes mellitus [237].

3.1.3 Pulse-volume recording

Physiology and implementation: Pulse volume recording (PVR) is an indirect measure of blood flow in the lower and upper extremities. The PVR is a functional test to examine total blood flow to the limb under investigation, rather than evaluating blood flow in specific blood vessels [46]. Since arterial inflow is pulsatile, it results in measurable changes in lower limb volume during each cardiac cycle [46]. PVR uses an air calibrated plethysmographic technique, whereby blood pressure cuffs are inflated on the lower limbs. The inflated cuffs compress the veins, but not the arteries, by inflating the cuffs to 65-70 mmHg. Tissue volume (limb volume) increases during systole and decreases during diastole, which is recorded as a PVR tracing. A transducer detects arterial pulsation from the pulsatility within each pulse, without any venous interference. This is converted into an analog signal that shows the amplitude and contour of the pulse wave during the cardiac cycle.

Clinical use and interpretation: In the present thesis, ankle PVR has been used as a screening tool for all participants to evaluate peripheral circulation in order to differentiate between healthy and diseased vascular physiology.

PVR is commonly used as an initial test to evaluate whether a patient's symptoms are associated with poor blood flow, in order to establish the presence of lower limb PAD. It is also used to monitor limb circulation after a revascularization intervention [46]. PVR is a valuable tool to evaluate small vessel disease when used on the feet and to evaluate blood flow in individuals with incompressible vessels, where ABPIs are spuriously elevated [46]. A PVR recording in a healthy individual with no flow-limiting disease proximal or beneath the

blood pressure cuff is typically displayed as a pulse with a brisk upstroke, sharp systolic peak, gradual downstroke with a dicrotic notch, and a gradual diastolic runoff [238]. The PVR amplitude is proportional to total inflow and pulse pressure in the measured limb segment. Local pulse pressure reflects the amplitude and is reduced with arterial occlusion proximal to the area of measurement [238]. As a result, reduced amplitude indicates greater proximal obstruction [238].

Several factors are likely to impact PVR blood flow measurements (amplitude and waveform). These include low cardiac stroke volume, blood pressure, (proximal) arterial disease, changes in body and local ambient temperature, inflammation, patients' positioning and movement, medication, edema, vitamins, and other factors. These factors may induce vasoconstriction or vasodilation of the arterial microcirculation.

PVR is a qualitative, not a quantitative measure of circulation, with less accuracy in more distal limb segments and in determining anatomical PAD localization [46]. In incompressible vessels (e.g. in elderly or diabetes patients), ABPI values may be artificially elevated as the vessels' rigidity masks lower extremity stenosis. As a result, ABPI measurements may fail to detect lower extremity PAD. It is therefore useful in such cases to confirm the presence of PAD using PVR [46].

One study compared the validity of ABPI and PVR measurements to detect PAD in 205 patients, verified by ultrasound duplex scans [239]. PVR sensitivity was 97 %, specificity 81 %, and overall accuracy 85 %. The sensitivity, specificity and accuracy of ABPI were 79 %, 91 %, and 88 %, respectively. The combined sensitivity of ABPI and PVR was 100 %, specificity 76 %, and overall accuracy 85 % [239]. While ABPI seems to provide excellent specificity, PVR has excellent sensitivity [239]. Together these two methods are useful tools to establish a differential diagnosis, and by extension, complete an initial diagnosis of lower extremity PAD. Although PVR is a cost-effective and useful screening tool, the method is limited by the lack of ability to specifically locate the anatomic location and degree of PAD [46].

3.1.4 Skin perfusion pressure

Physiology and implementation: Skin perfusion pressure is a non-invasive technique that uses a laser Doppler flux and a pressure cuff to assess reactive hyperemia. Skin perfusion

pressure is a measure of the pressure at which cutaneous blood flow (capillary flux) first returns during the controlled release of occlusive cuff pressure. Investigations have found a strong correlation between SPP, toe-brachial pressure, and the healing rate of ischemic ulcers [240]

Clinical use and interpretation: ABPI is an optimal method to estimate macrocirculation. As noted above, however, patients with calcified peripheral arteries (i.e. in elderly, and in patients with diabetes mellitus or chronic kidney disease) may register falsely elevated ABPI values [230]. In order to evaluate peripheral circulation in calcified vessels, including microcirculation during the return of blood flow to capillaries, SPP measurements are a useful complementary diagnostic tool [241] .

Several studies report cut-off values of ≥30 as indicative of critical limb ischemia and unfavorable for ulcer healing to occur [240, 242, 243]. Further, Yamada et al. [240] found that a minimum of 40 mmHg SPP was necessary for ulcer healing to occur. SPP measures have been found to predict improvement in tissue perfusion after revascularization in patients with critical limb ischemia [242]. Additionally, SPP is more reliable than TcPO2, toe-brachial pressure and ankle systolic blood pressure in predicting ulcer healing [240].

In the present thesis, SPP has been used to evaluate peripheral circulation in patients with severe PAD who underwent INP treatment for eight weeks (Paper II).

3.1.5 Laser Doppler fluxmetry

Physiology and implementation: Laser Doppler fluxmetry (LDF) is a noninvasive technique to measure microvascular blood cell flux (flow) by detecting cell movement in the microcirculation [244]. LDF is an optical (i.e. the use of light) technique [245] to estimate microcirculation. When the laser beam is directed towards the tissue, the following processes take place: reflection, transmission, absorption, and scattering [245]. The technique is based on the Doppler principle mentioned above, which measures microcirculatory perfusion from the amount and frequency shifts of laser light backscattered photons (mainly reflecting red blood cell motion) [246]. With Doppler fluxmetry, laser light hits moving particles such as red blood cells and undergoes change in wavelength. Laser light that hits non-moving particles remains unchanged [245]. The extent and frequency distribution of these changes in wavelength are directly related to the number and velocity of the blood cells in the sample volume. The

measurement depth is approximately 0.5-1 mm in normal skin using standard fiber separation (0.25 mm) and a 780nm wavelength laser [247]. This is sufficient to measure perfusion in arteriovenous anastomoses [248]. LDF measurements are expressed in arbitrary units (AU) or perfusion units (PU), as no laser Doppler instruments provide absolute perfusion values [246].

Clinical use and interpretation: In the present project, the PeriFlux System 4000 (Perimed, Järfalla, Sweden) was used during all laboratory measurements of acral skin blood perfusion during the INP experiments.

3.1.6 Transcutaneous Oxygen Pressure

Physiology and implementation: Transcutaneous oxygen pressure ($TcPO_2$) is a noninvasive way to measure oxygen tension in the skin. Suggested applications are evaluating wound healing potential, screening for vascular disease and revascularization success, predicting amputation level and predicting the benefit of hyperbaric oxygen therapy. $TcPO_2$ is a non-invasive measure of the local oxygen content of a small defined area of microcirculation during local hyperemia [249], reflecting the quantity of oxygen that has diffused from the capillaries and is available for skin microcirculation [250].

Clinical use and interpretation: At sea level the pressure is 760 mmHg (101.3 kPa), and 21 % of this is oxygen. Inspired partial pressure of oxygen is therefore 160 mmHg (21.3 kPa; 21 % of 760 mmHg). In the lungs, oxygen gets diluted with water and carbon dioxide. The pO_2 in the arteries is approximately 100 mmHg. It is transported with the vasculature to the capillaries and finally the tissues, where the $TcPO_2$ is usually around 70 mmHg (9.3 kPa) (depending on tissues investigated). For example, values are usually lower in the foot compared to the leg [249] with average of >50 mmHg (6.7 kPa) considered a normal value in healthy individuals breathing normobaric air. Low critical values of $TcPO_2$ are <40 mmHg (5.3 kPa). Wound healing is a complex process. Tissue hypoxia as well as nutritional status, diabetes status, medication, etc. interferes with the healing. It is easier to determine a value below which a wound will not heal than to identify a value above which a wound is predicted to heal. However, the value depends on other factors, disease and environmental, such as whether the individual has pulmonary disease, heart failure, peripheral vascular disease or if the individual resides at altitude when the measurements are performed. Additionally, edema or inflammation may constitute a barrier to oxygen diffusion to the electrode, also affecting the $TcPO_2$ values.

Transcutaneous oxygen pressure ($TcPO_2$) in paper II was measured during the whole experiment with a transcutaneous oximeter (PeriFlux PF 5010/5050 Pressure Unit; Perimed, Stockholm, Sweden). Prior to taking the measurements, the unit was calibrated in room air according to the manufacturer's instructions, and the electrode was heated to 43°C to induce increased cutaneous blood flow. A self-adhesive fixation ring (TC550, Perimed AB, Stockholm, Sweden) was applied to the skin site at the dorsum of the foot in close proximity to the area of the extensor hallucis brevis muscle. The hole was filled with contact liquid (TC560, Radiometer, Perimed AB, Stockholm, Sweden), and the $TcPO_2$ electrode was screwed into the fixation ring.

3.1.7 Photographic wound assessment tool

Physiology and implementation: A photographic wound assessment tool (PWAT) is tool to evaluate wound photographs with eight items that describe the appearance of the wound bed, peri-ulcer skin viability, and wound edges in order to evaluate a variety of ulcers; diabetic, venous/arterial and pressure-related etiology [251]. There are many tools developed and used by clinicians to assess wound evaluations, such as the Leg Ulcer Measurement Tool (LUMT) [252] and Pressure Ulcer Scale for Healing (PUSH) [253], which have been found to provide valid and reliable measurements of wound appearance when used by an experienced clinician working at the patient's bedside [252, 253]. The PWAT, in contrast, has also been shown to have excellent agreement with assessments using digital wound photographs and bedside assessments while directly visualizing the ulcer (ICC = 0.89) [251].

Clinical use and interpretation: The revised PWAT consist of eight domains, as opposed to six in the previous version [254], each scored on a five-point scale from zero to four. This yield a maximum score of 32, where zero represents a completely healed ulcer [251]. The PWAT has been shown to detect changes between healing and non-healing ulcers over time [254].

3.7 Laboratory experiments

All experiments on acute blood flow (paper I-IV) were conducted in a quiet, temperature-controlled environment (~25°C) controlled by a mechanical thermostat (Technibel air conditioner, Trévoux CEDEX, France) to reduce sympathetic stress that could create artifacts [255]. Prior to all experiments, participants were asked not to drink alcohol, coffee or tea, and not to exercise the day of the test.

3.8 Beat-by-beat data sampling and signal acquisition

In the experimental studies on acute blood flow (Paper I-Paper IV), the physiological parameters were sampled with a custom-made computer program for registration of real-time physiological data acquisition (REGIST3, Morten Eriksen, University of Oslo). The R-R intervals were sampled from a three-lead electrocardiogram (SD100, GE Vingmend, Horten, Norway). Beat-to-beat values of the cardiovascular parameter (flow velocity, flux, MAP) were calculated from the R-wave in each heart cycle. This makes it possible to calculate one value for each physiological parameter, and to follow and examine blood flow velocities of the foot arteries during individual heart beats before, during, and after periods of negative pressure. Analog signals from all sensors were sampled at 300 Hz and averaged for each heartbeat gated by ECG R-waves in each cardiac cycle using the custom-made software REGIST3. All physiological data were sampled and stored at 300 Hz. For further analysis, data were down-sampled to 2 Hz. The signals were then sent a personal computer. All data were imported to Microsoft® Excel (Microsoft Office 2010 for Windows; Microsoft Corp., Redmond, WA, USA) for further analysis. All participants' identities were protected with a unique code in order to de-identify the data before final statistical analysis.

Figure 8 shows an example from the program during one experiment, displaying all the physiological variables. The data was captured during six 120s experiments repeated twice (study I) and during one 20 min experiment for each participant (Study II, III and IV).

In paper I, the 120s negative pressure sequences were administered in a randomized order using an Excel random number generator (Microsoft Office 2010 for Windows). Finger arterial blood pressure was measured continuously and non-invasively by the photoplethysmographic volume-clamp method (Finometer; FMS Finapres Medical Systems BV, Amsterdam, The Netherlands). The method has been validated for research purposes [256]. Before attachment of the finger pressure cuff, calibration with an upper arm cuff was performed by an automated sphygmomanometer (Solar 8000i, GE-Marquette Medical Systems, Inc., Milwaukee, USA). The finger pressure cuff was attached on the middle phalanx of the third finger of the right hand before the experiment. In the volume-clamp method, the finger artery beneath the cuff is "clamped" (i.e. the cuff diameter is kept constant by applying counter-pressure during changes in arterial pressure throughout the cardiac cycle). The pressure changes are measured by infrared phot-plethysmograph in the cuff, and transformed to finger arterial blood pressure [257]. The method is sensitive to change in

hydrostatic pressure, and care was therefore taken to ensure that the patient did not move during the experiments. After attachment of probes and ECG, the participants' bodies were covered with a light blanket, and their feet were covered with loose, non-elastic wool socks to keep them warm. All experiments were performed in room temperatures above 24°C to facilitate highest fluctuation frequencies and largest amplitude (optimal conditions) in the acral cutaneous blood flow measurements [258]. The participants' thermoneutral zone was supported by observed observed temporal variations in flow (flowmotion) at rest reflected by high fluctuation frequencies in the acral cutaneous blood flow of both feet and large fluctuations in arterial blood flow velocity.

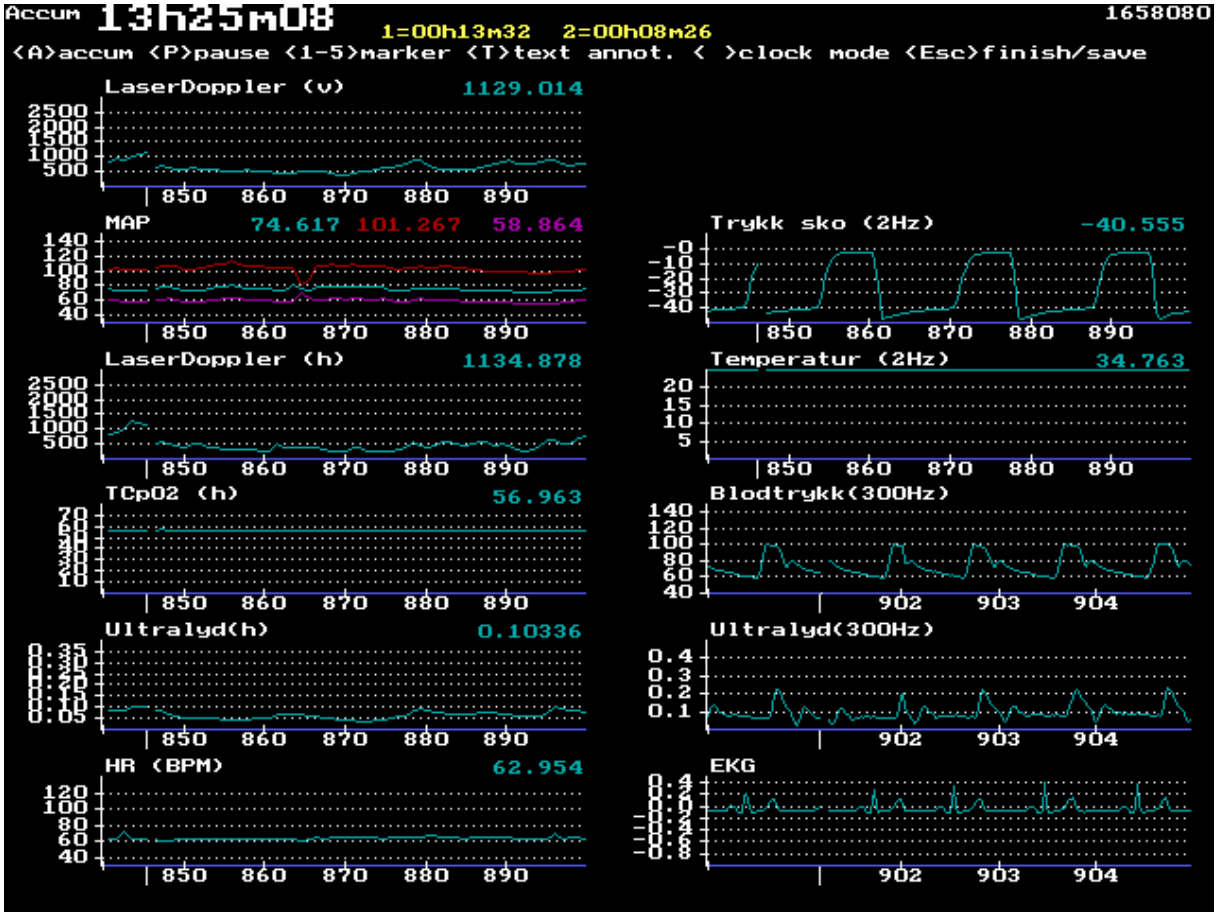

Figure 8. Screenshot from the program REGIST showing all the physiological parameters described in Paper I-IV.

Panels are from top left laser Doppler flux left foot, mean arterial pressure (MAP), laser Doppler flux right foot, transcutaneous oxygen pressure (TcPO2), ultrasound, heart rate (HR), pressure chamber, skin temperature, blood pressure (300 Hz sampling frequency), ultrasound (300 Hz sampling frequency), ECG.

3.9 The experimental set-up in paper I-IV

After vascular assessments, the participants were comfortably clothed and seated with an approximate angle of 130° in their knee and hip joints in a chair for 20 min before the experiment. After attachment of probes on the foot, the patient's body and legs were covered with a blanket and both feet were covered with loose, non-elastic wool socks to keep the feet warm and under approximately constant temperature during the course of the experiment. The test leg was placed in a rigid molded polyethylene custom-made subatmospheric pressure chamber coupled to a pressure control system (FlowOx™; Otivio AS, Oslo, Norway). The contralateral leg was placed outside the subatmospheric pressure chamber. Internal padding in the subatmospheric pressure chamber allowed for insertion of the leg with probes attached, preventing pressure points on the skin of the leg and foot with only the posterior part of the lower leg in contact with the internal padding. The researcher ensured that the participant's foot moved freely inside the subatmospheric pressure chamber (Figure 9). The subatmospheric pressure chamber was sealed just below the knee with a thermoplastic elastomer (TPS-SEBS). Figure 9 depicts the experimental set-up used in paper I-IV to measure changes in acute foot macro- and microcirculation during INP.

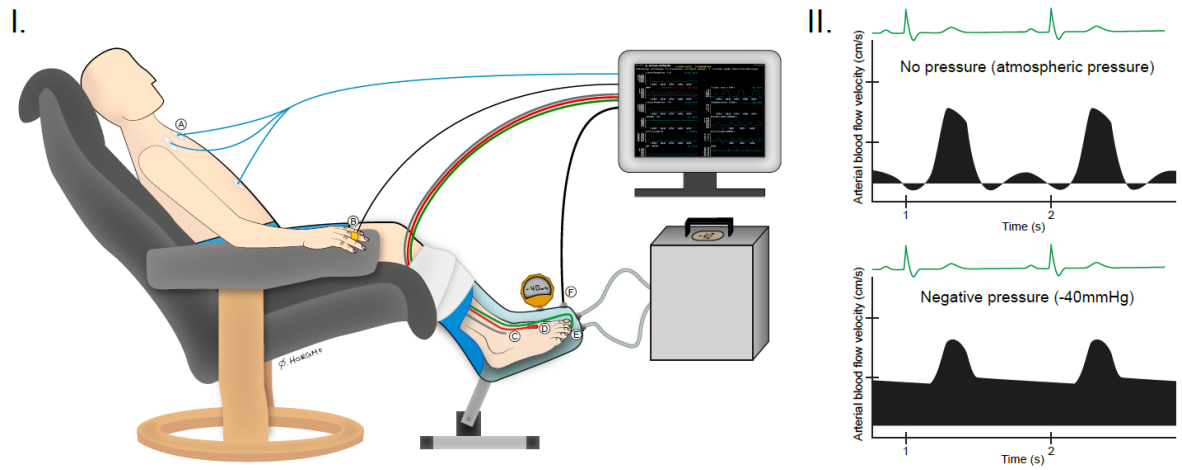

Figure 9. Illustration of the experimental setup in Paper I-IV.

I: The participant's lower leg and foot (test leg) was placed in the custom-made subatmospheric pressure chamber interfaced with the pressure control system. The contralateral leg was placed outside the subatmospheric pressure chamber. A: Three-lead electrocardiography; B: Finometer; C: Skin temperature probe; D: Ultrasound Doppler probe; E: Laser Doppler flux probe; F: Pressure transducer from subatmospheric pressure chamber interfaced with the computer. An additional external calibrated pressure gauge (Fluke, 700G Series, Everett, WA, USA) was connected to the subatmospheric pressure chamber in order to calibrate the data program (REGIST 3) before each experiment. II: Example of typical arterial blood flow velocity response in the dorsal pedis artery/tibial posterior artery during application of INP, with the diastolic velocities seemingly contributing the most to the observed increased blood flow velocity. Illustration: Øystein H. Horgmo, University of Oslo.

3.10 Blood flow measured in large arteries during INP

In a separate experiment on six additional PAD patients (age range: 60-77 years) with stents in their superficial femoral arteries, stent blood flow was recorded during INP oscillations in order to evaluate blood flow in arteries with fixed diameters. INP was performed using cycles of 10s of -40mmHg negative pressure and 7s of atmospheric pressure as described above. We compared blood flow by measuring blood flow during six heartbeats before onset of negative pressure to the six first heartbeats after onset of negative pressure, using automated software (EchoPac PC, v.7.0.1, GE Healthcare, Horten, Norway). Blood flow was recorded with an ultrasound scanner using a 9 MHz Doppler probe (E9; GE Vingmed Ultrasound, Horten, Norway), multiplying the time-averaged velocity over six cardiac cycles by the cross-sectional area of the stents' inner diameter using electronic calipers, as previously described [259]. The rationale for examining the effect of INP on blood flow in large arteries with stents was to evaluate changes in blood flow in arteries with fixed diameter (in arteries located outside and proximal to the subatmospheric pressure chamber), and to examine the effect of INP on runoff through the stents.

3.11 Statistical analysis

In Paper I-IV, continuous variables are presented as mean (standard deviation) or median (range), and when appropriate, median with interquartile range (IQR). Statistical analyses were performed using the statistical package R with the "nlme"[260] and "multcomp"[261] add-on packages (R Foundation for Statistical Computing, Vienna, Austria).To account for repeated measurements within subjects, estimates and 95 % confidence intervals (CI) were calculated in a mixed effects model regression analysis with subject as a random effect. For all statistical tests, differences were considered significant when $P<0.05$.

3.11.2 Comparison of mean values between sequences – estimates of flow and flux

For each subject, mean arterial blood flow velocity (cm/s) and laser Doppler flux (AU) were calculated for each of the experimental sequences (baseline, INP and post-INP, respectively). To remove disturbances (artificial noise) in the transition zones, the first and last 10 s of each sequence were eliminated before analysis. Effects of the INP and post-INP sequences compared to baseline were evaluated in a mixed effects regression model with subject as a random effect, by assigning variables to each sequence.

3.11.3 Changes over time within one negative pressure cycle

In order to calculate the time-averaged arterial blood flow velocity of individual cardiac cycles, blood flow velocity values from each of the participants' heartbeats were analyzed by calculating the average of the blood flow velocity per heartbeat. To evaluate the effects of time within each sequence, the time of each observation (heartbeat) was rounded to the nearest second. To compare the change in arterial blood flow velocity and flux over time within one INP cycle (1 cycle=17s total), arterial blood flow velocity and flux were binned within each second after the onset of negative pressure. Baseline and post-INP measures were calculated using the average flow velocity and flux values during the respective sequences. In study III and IV, additional post-INP sequence analyses were performed in order to examine the intermediate effects of INP (defined herein as the effects on blood flow the first minutes after termination of INP). The flow and flux effect of each second after the start of negative pressure compared to mean flow velocity during the baseline sequences was evaluated in a mixed effects model with subject as a random effect by assigning variables to each second.

In Paper IV, we estimated the maximum blood flow by cross-validation. This was performed to avoid potential errors by picking random peaks in blood flow as the maximum, when the same data is used to both locate the maximum blood flow and estimate its magnitude. Cross-validation was performed by randomly dividing 24 subjects into eight groups. Thereafter, we randomly pinpointed the time of maximum flow and flux in seconds for the 21 subjects (seven groups). We then estimated flow and flux magnitude as the average flow and flux values for the given time (in seconds) among the remaining group (three subjects). The process was repeated for all groups, with flow and flux magnitude estimated as the average maximum flow and flux value across all cross-validation samples. We then performed 10,000 bootstrapping replications of the patient groups to estimate their respective confidence intervals. Note that we did not use this process in calculating the estimates of changes over time within a single pressure cycle (Figure 14 and Figure 3 in Paper IV). This explains the discrepancy between peak blood flow in that estimate and in the cross-validated maximal blood flow.

3.11.4 Cumulative up-and-down in arterial blood flow velocity

To evaluate the fluctuations in arterial blood flow velocity during INP relative to baseline and post-INP sequences, the differences between the average blood flow velocity during each

heartbeat and the previous were calculated. The sum of the absolute values of these differences for each subject was calculated within each sequence, and averaged per minute. This gave the cumulative up-and-down variations in blood flow velocity per minute. By assigning variables to each sequence (baseline, INP and post-INP), we compared the cumulative changes within each sequence in a mixed effects model. Estimates and confidence intervals were also calculated.

3.11.5 The effect of treatment allocation on ulcer healing

The effect of INP+ SWC vs. SWC alone on % delta WSA and PWAT scores before and after finishing period 1 of the crossover study was assessed with statistical software using the chi-square test with Agresti-Caffo corrections (Stata, version 11, StataCorp LP, College Station, TX). We used the chi-square test with Agresti-Caffo corrections to find *P*-values (of treatment effect) between the treatment groups, INP+SWC vs. SWC alone.

Since normal distribution could not be assumed from our data, we have performed bootstrapping to find confidence intervals on the treatment effects. We performed 1,000,000 bootstrapping replications (percentile bootstrapping) to calculate confidence intervals. Bootstrapping was performed in the statistical package R using the package "Library boot" (version 3.3.3, R Foundation for Statistical Computing, Vienna, Austria). The statistical analysis was performed by one of the co-authors (HW-F).

3.11.6 Sample size calculations

Papers I-IV:

The number of participants needed for the experimental studies of the project was estimated on blood flow in the main artery of the foot (dorsal pedis artery), the project's main outcome variable. Based on pilot tests on patients with PAD [262], a mean baseline flow at 28 mL/min with a standard deviation at ±12 mL/min was expected in the dorsalis pedis artery. A 30 % increase in blood flow in the dorsal pedis artery compared to baseline was considered a clinically significant increase in blood flow. According to the power calculation, a minimum of 18 subjects need to be included in each experiment, given a power of 80 % and a 5 % significance level.

Paper V:

The expected effect of INP on ulcer healing was unknown prior to our study. We therefore performed a pilot RCT study which would guide the necessary sample-size calculations for larger randomized controlled trials in the future. Based on power calculations with dichotomous outcome of >or<15 % healing rate in eight weeks, we assumed that 80 % in the INP+SWC group would show >15 % healing rate, and 20 % of the patients in the SWC only group would show >15 % healing rate. Based upon these calculations, a total of 26 patients was deemed necessary with a power of 80 % and a 5 % significance level. We therefore aimed to allocate 13 patients in each group by the end of December 2016, with block sizes ranged from 2 to 4 using Stata's ralloc command (Stata, version 11, StataCorp LP, College Station TX).

4 RESULTS

4.1 Paper I

Title: *Application of intermittent negative pressure on the lower extremity and its effect on macro- and microcirculation in the foot of healthy volunteers.*

What this study adds and the new findings

We described the cardiovascular responses to four different sequences, each with different oscillation patterns of mild (-40 mmHg) negative pressure applied to the lower leg and foot in 23 healthy volunteers. Our beat-to-beat analyses demonstrated an immediate and significant effect of negative pressure on foot macro- and microcirculation. These results showed that INP induced fluctuations in blood flow velocity and cutaneous blood flow of the foot throughout the INP sequences, without any clinically significant changes in heart rate (HR) or mean arterial pressure (MAP). Specifically, blood flow velocity reached a maximum of 44 % increase above baseline (95 % CI 33 % to 55 %, $P<0.001$) at 4s after the onset of negative pressure, with similar fluctuations observed in pulp cutaneous blood flow of the foot. The average blood flow velocity increased 8 % during the 10 s-7 s sequence compared to average flow velocity during the baseline sequence (atmospheric pressure). In contrast, constant negative pressure significantly reduced arterial blood flow velocity of the foot by 12 % compared to the baseline sequence (see the below paragraph). Figure 10, panel b shows the changes in arterial blood flow velocity during a 2 min constant negative pressure sequence.

We observed small changes in average arterial blood flow velocity during all of the INP sequences, except during the 15s-15s sequence, during which it did not increase. Mean arterial foot blood flow velocity during the baseline sequence (no pressure) was 8.9 cm/s (95 % CI 6.4 cm/s to 11.4 cm/s). The mean changes in arterial foot blood flow velocity during INP sequences of 10 s-7 s, 15 s-15 s and 30 s-30 s (negative pressure on/off, respectively) were (95 % CI) 0.7 cm/s (95 % CI 0.51 cm/s to 0.89 cm/s, $P<0.001$), -0.11 cm/s (95 % CI -0.31 cm/s to 0.08 cm/s, $P=0.41$) and 1.18 cm/s (95 % CI 0.99 cm/s to 1.37 cm/s, $P<0.001$), respectively. During 2-min constant negative pressure sequences, mean arterial foot blood flow velocity decreased [-1.1 cm/s (-1.3 cm/s to -0.9 cm/s, $P<0.001$)] (Paper I and Figure 10, panel b.), along with foot skin temperature and pulp skin blood flow.

To our knowledge, this is the first description of the isolated effects of different negative pressure oscillation sequences on foot macro- and microcirculation. Also, the participants reported no unpleasant sensations or pain during the experiment (Paper I, Table 1). The increased arterial foot blood flow velocity during negative pressure oscillations was due to INP-induced increases in arterial blood flow fluctuations (flow pulsatility). Figure 10 and Figure 11 show how arterial blood flow velocity fluctuates below and above normalized baseline blood flow. Figure 11 displays the raw data for all 23 healthy subjects, revealing large variations in the effect of INP on arterial blood flow velocity across participants.

All experimental sequences had negative pressure for a minimum of 10s. For the first 10s of all negative pressure sequences (aggregated for the whole experiment in all participants), initial blood flow increased. The initial flow velocity response during the first negative pressure period was similar for all four different negative pressure sequences: 30 s-30 s, 15 s-15 s, 10 s-7 s and 2-min constant negative pressure.

In Paper 1, all sequences were performed twice (2 sets). The first and second sets are displayed separately, as they were used to locate and calculate the maximal effect over time. The maximal effects were located at 4 s and 5 s for the first and second sets, respectively. The calculated effects of time corresponded to an increase of 57 % and 59 % for the first and second repetitions, respectively, giving an estimated maximal effect of time of 58 % increase compared to flow velocities during the 2-min baseline (no pressure) sequences (Figure 12). The initial flow response when calculating the aggregated response for all pressure cycles during the whole 120 s experiments was, however, slightly smaller (44 %). This is because the flow response for the first pressures cycles initially had the highest peaks, as can be observed with the naked eye in Figure 10.

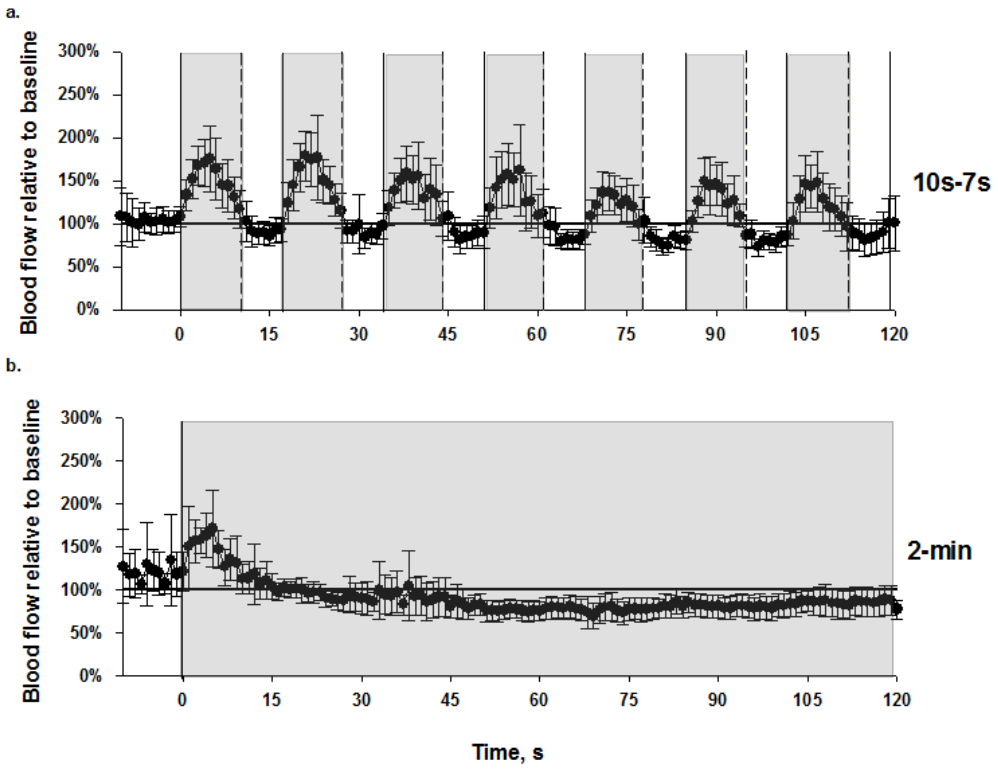

Figure 10. Arterial blood flow velocity in the foot arteries during 120 s sequences.

a, arterial blood flow velocity during application of INP pattern alternating 10 s at -40 mmHg and 7 s at atmospheric pressure; b, arterial blood flow velocity during application of constant negative pressure of -40 mmHg. Adapted from Sundby et al. Physiol Rep. 2016 Sep;4(17). pii: e12911. doi: 10.14814/phy2.12911 (Paper I).

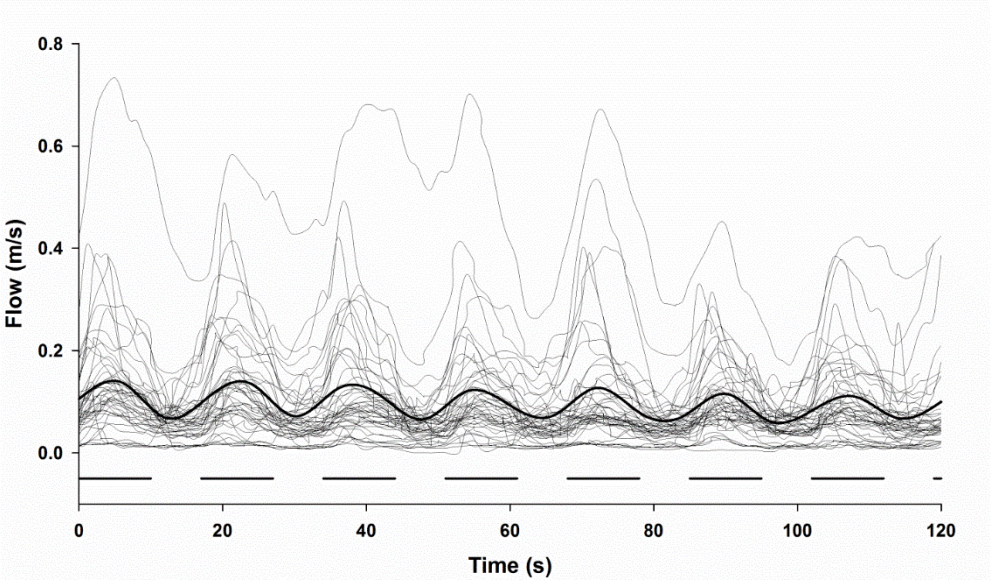

Figure 11. Effect of time in the 10 s-7 s INP pattern during the 120 s sequence in healthy volunteers.

Flow velocity data in all 23 healthy participants are presented as thin lines (raw data). Predicted values are presented in spline regression as thick lines. Negative pressure is denoted by black lines (bottom). The empty spaces represent atmospheric pressure.

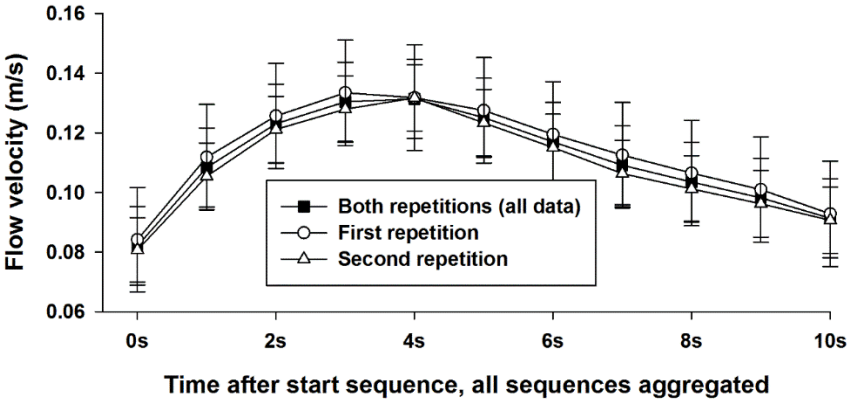

Figure 12. Effect of time for the first 10 s of all negative pressure sequences aggregated.

Error bars are standard error. To estimate the maximal effect (peak) of INP on blood flow, the first 10s of all four sequences were aggregated. In order to avoid using the same data to both locate the maximal flow and estimate its magnitude, we used the first repetition to locate the time of maximal flow and the second repetition to estimate its magnitude, and vice versa. The mean of these two calculations was then used to estimate the maximal effect of INP on blood flow velocity. The largest effect of time was found 4 s after the onset of negative pressure for the first repetition. For the second repetition, the largest effect was found 5 s after the onset of negative pressure. The calculated effects of time corresponded to an increase of 45 % and 43 % for the first and second repetition, respectively, giving an estimated maximal effect of time of 44 % (95 % CI 33 % to 55 %) increase.

How this study may have an impact on clinical practice in the future

Increasing macro- and microcirculation flow to the tissues means that the availability of oxygen and nutrients to the cells increases. In patients with insufficient blood flow, this may increase the oxygen supply/demand ratio, which may improve wound healing and reduce infection risk in ulcers with low tissue perfusion. More research is needed to determine whether the observed effect will result in long-lasting changes in collateral circulation and improved tissue perfusion in relevant patient groups (patients with lower limb ischemia and/or chronic leg and foot ulcers). The INP method may theoretically induce increase in shear stress, thereby eliciting beneficial changes to the endothelium. Increased blood flow and flow-induced shear stress may potentially increase patency after revascularization.

4.2 Paper II

Title: *The effects of intermittent negative pressure on the lower extremities' peripheral circulation and wound healing in four patients with lower limb ischemia and hard-to-heal leg ulcers: a case report.*

What this study adds and the new findings

The study's novel findings suggest that treatment with INP ~2-hours per day for eight weeks may improve wound healing and induce long-lasting circulatory effects in patients with severe PAD and hard-to-heal leg and foot ulcers. The increased ABPIs found in our paper (II) may indicate that INP improved collateral circulation in patients with severe PAD and lower limb ulcers. We also showed for the first time — in an experiment on one of the patients — that blood flow velocity and pulp skin blood flow, together with transcutaneous oxygen pressure of the foot, increased during INP compared to a baseline period at atmospheric pressure.

How this study may have an impact on clinical practice in the future

This exploratory case study suggests that that mild negative pressure (-40 mmHg) applied intermittently (alternating 10 s on and 7 s off) may induce beneficial clinical changes to promote the healing of ischemic tissue. These findings should be examined in adequately powered RCTs.

4.3 Paper III

Title: *The acute effects of lower limb intermittent negative pressure on foot macro- and microcirculation in patients with peripheral arterial disease.*

What this study adds and the new findings

The results showed that INP applied to the lower leg and foot increases foot macro- and microcirculatory flow pulsatility in patients with PAD intermittent claudication. Further, our paper showed that application of INP increased mean arterial blood flow velocity in the foot, and that this increase was sustained for at least 5-min after termination of INP. This increased blood flow velocity was observed without any significant differences in INP induced flow velocity fluctuations between post-INP and baseline (Figure 13). This may indicate that INP

has a prolonged effect on blood flow. These results are encouraging considering the relatively short duration of INP treatment (10 min).

How this study may have an impact on clinical practice in the future

Increased arterial and skin blood flow to the tissues may have beneficial physiological effects in ischemic limbs, such as increased wound healing, reduced pain and improved ambulatory capacity. The clinical effects of this of augmented INP-induced flow in PAD patients should be investigated in an RCT.

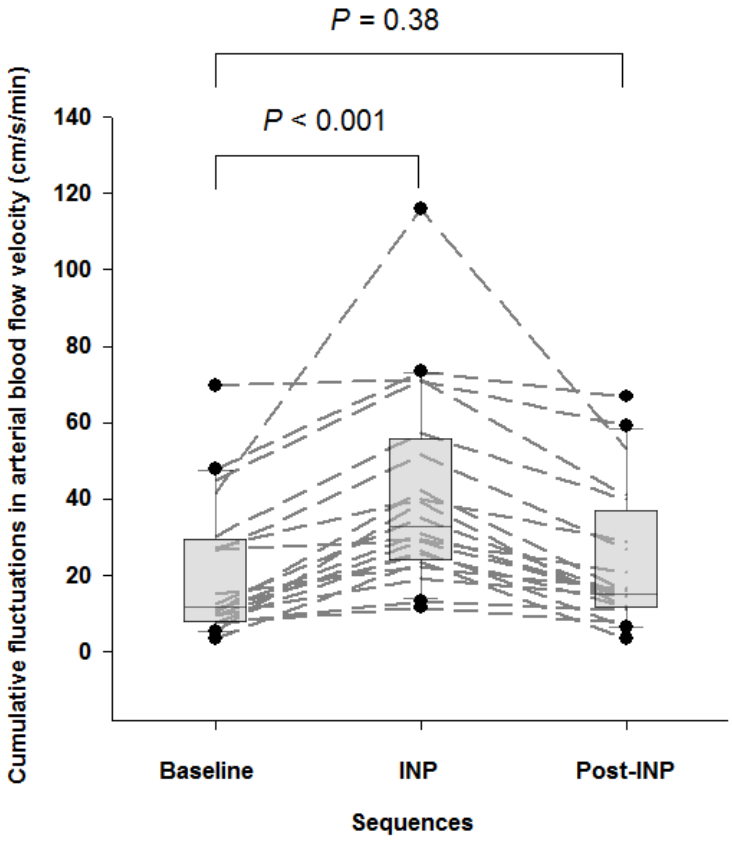

Figure 13. Boxplot of fluctuations in arterial foot blood flow velocities between sequences.

The figure shows fluctuations (cumulative up-and-down) of PAD patients' (n=20) arterial blood flow velocities during the baseline (no pressure), INP, and post-INP (no pressure) sequences.
The cumulative up-and-down are cm/s/min. The whiskers are 10th and 90th percentiles. A linear mixed-effects model showed a significant effect of INP compared to baseline (P<0.001), while there were no significant differences between baseline and post-INP (P=0.38).

4.4 Paper IV

Title: *Intermittent negative pressure applied to the lower limb increases foot macro- and microcirculatory flow pulsatility in people with spinal cord injury*.

What this study adds and the new findings

This study's novel finding is that INP applied to the lower leg and foot increased arterial blood flow pulsatility of the foot compared to baseline in individuals with SCI (Figure 14 and Figure 4 in Paper IV). In the people with SCI, blood pressure and heart rate did not change from baseline to INP [-0.6 mmHg (95 % CI -2.8 mmHg to 1.5 mmHg), P=0.55] and -0.6 beats/min (95 % CI -1.3 beats/min to 0.2 beats/min, *P*=0.16), respectively. In sum, arterial blood flow pulsatility increased without changes in central hemodynamics.

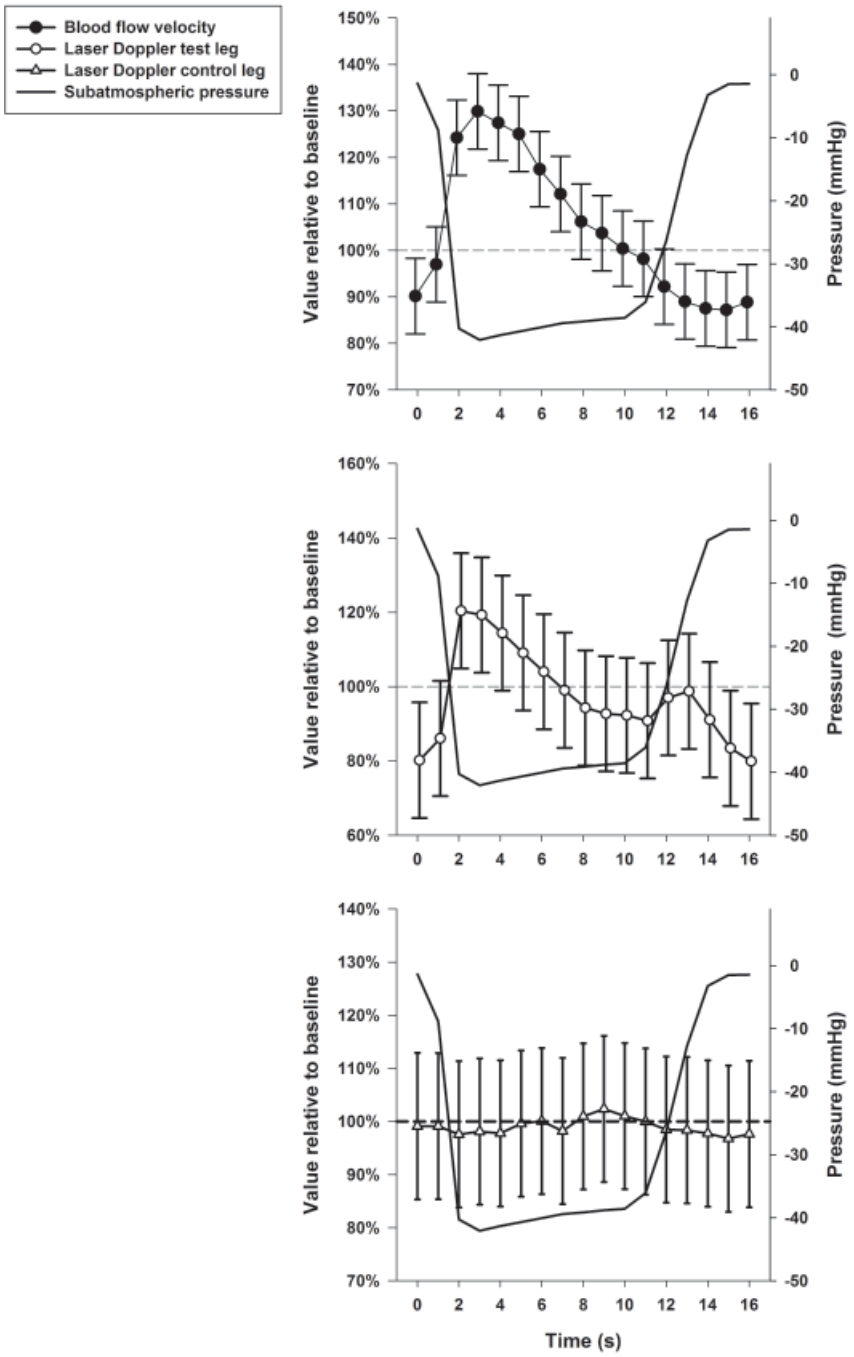

Figure 14. The effects of blood flow velocity and laser Doppler flux in the test and the control leg, respectively, for the first 17s (one pressure cycle) after onset of negative pressure among all SCI participants.

Note that zero denotes onset of negative pressure. The upper panel's black circles denote arterial blood flow velocity (cm/s). The middle panel's white circles denote laser Doppler flux in test leg (exposed to INP). The lower panel's open triangles denote laser Doppler flux in the foot outside the pressure chamber. One pressure cycle equals 10 s of 40 mmHg subatmospheric pressure and 7 s of atmospheric pressure. Subatmospheric pressure chamber (mmHg, right y-axis). All are mean values with 95 % confidence intervals relative to the mean baseline sequence.

How this study may have an impact on clinical practice in the future

Skin breakdown of the lower limbs commonly affects individuals with chronic SCI. In addition to deficient sensitivity and immobility, many patients with chronic SCI have increased atherosclerotic risk factors: many are sedentary, elderly, have disturbed lipid and carbohydrate metabolism, and/or are smokers [263]. Poor skin blood flow has been shown to be present in individuals with chronic SCI who spend many hours in a wheelchair [264]. The insufficient skin circulation has been suggested as one important factor for poor healing of lower extremity ulcers in individuals with chronic SCI [264]. Therefore, inducing increased circulation to the muscles and skin may facilitate healing of chronic ulcers in individuals with SCI.

Post-hoc analysis in for the SCI population in paper IV, indicated the people with SCI with lower ABPI also had greater potential to increase blood flow during INP. The post-hoc analysis showed that an increase in ABPI of 0.1 was associated with a 0.33 cm/s (95 % CI 0.035 cm/s to 0.63 cm/s, *P*=0.034) smaller increase in blood flow velocity from baseline to INP (Figure 15).

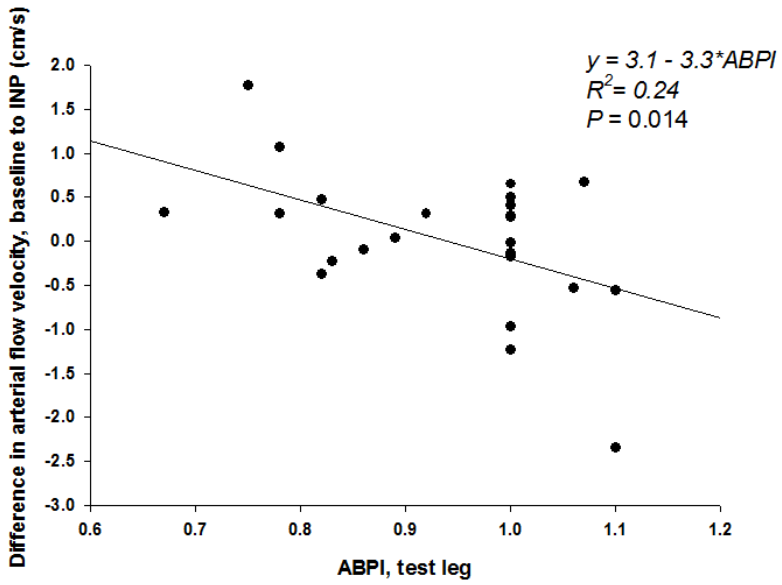

Figure 15. The relationship between ABPI and arterial flow velocity difference between baseline and INP sequences.

The figure shows a significant inverse relationship between ABPI and arterial blood flow velocity difference between baseline and INP sequences (post-hoc analysis). The calculations are performed in a linear regression model without the post-INP sequence included in the analysis (explaining the different P-value from the mixed regression model used to calculate post hoc analysis).

4.5 Paper V

Title: *The effects of intermittent negative pressure applied to the lower limb on leg and foot ulcer healing in patients with spinal cord injury: a clinical crossover pilot study.*

What this study adds and the new findings

Most of the patients adhered to a 2-hour daily INP protocol for eight weeks. An increase in WSA was observed in 4 of 4 patients for INP+SWC vs. 3 of 5 patients for SWC alone (P=0.31). PWAT improvement was observed in 4 of 4 patients for INP+SWC vs. 2 f 5 patients for SWC alone (P=0.13). Using bootstrapping to calculate confidence intervals after treatment in the first period, the expected treatment effect for WSA after INP+SWC treatment compared to SWC treatment alone was 42 % (95% CI -168 % to 73 %). Observed differences in PWAT after INP+SWC treatment compared to SWC treatment alone was 11% (95% CI -151 % to 45 %). Due to the large uncertainty in our small dataset, combined with the fact that an ulcer may be indefinite worse, but never can heal more than 100 %, confidence intervals are in practice strong asymmetric on the percentage scale. In sum, because of the low sample size in this pilot study, the results need to be interpreted with caution.

This study suggests that repetitive use of INP approximately 2 hours per day combined with SWC may have an additive effect on ulcer healing in SCI patients with chronic leg and foot ulcers.

Two of nine patients did not complete the crossover study protocol. One patient acquired a skin infection during period one. When he began INP+SWC treatment in period two, the infection worsened. INP-induced increases in lower limb circulation may in theory exacerbate a skin infection, which involves the cutaneous lymphatics. Future studies should take care to screen for skin infection before inclusion. The other patient started to bleed after one INP treatment and chose not to continue.

In conclusion, the study demonstrates that INP is a feasible home-based treatment for SCI patients and should be examined in an adequately powered RCT.

How this study may have an impact on clinical practice in the future

Pressure ulcers are one of the most frequent secondary complications in people with SCI [140]. The cost of treating pressure ulcers in SCI individuals in particular is substantial. For example, Stroupe et al. (2011) [265] estimated the annual total health care cost in veterans with SCI and pressure ulcers compared to those without ulcers to be 100,935 USD vs. 27,914 USD ($P<0.001$). The prevalence of pressure ulcers among individuals with chronic SCI varies in the literature between approximately 15-35 % [266, 267]. About 39 % of US veterans have reported to have received health care for pressure ulcers during a three-year follow-up period [141]. Part of the extra costs related to SCI and ulcers are the additional costs related to wound care due to the need for extra assistance, transportation etc. SCI Individuals with ulcers have shown to exhibit more paralysis. They also tend to be older and have lower mobility, and are therefore more dependent on others to complete activities of daily living [138]. Avoiding complications related to surgery and amputations, as well as improving ulcer healing may therefore considerably improve an individual's overall quality of life and physical function and reduce the costs to society. Many people with SCI experience recurrent ulcers and slow healing rate compared to able-bodied individuals [268, 269]. They may have poor blood circulation to the tissue, at least partly, explaining the slow healing tendency [264]. Improving circulation of the small vessels to the cutaneous tissue may therefore have impact on the healing rate for people with SCI.

4.6 Supplemental data on blood flow measures in arterial stents with fixed diameter in PAD patients (unpublished data)

Figure 16 shows an example of the changes in arterial blood flow in a PAD patient's stent during INP. In this particular patient (age: 77 years; ABPI, right side with stent: 0.87; ABPI, contralateral side: 0.80), the stent diameter in the right superficial femoral artery was 4.0 mm). Blood flow increased by ~77 % during the negative pressure period compared to no pressure (bracket A and bracket B, Figure 16), with the diastolic velocities seemingly contributing the most (bracket B).

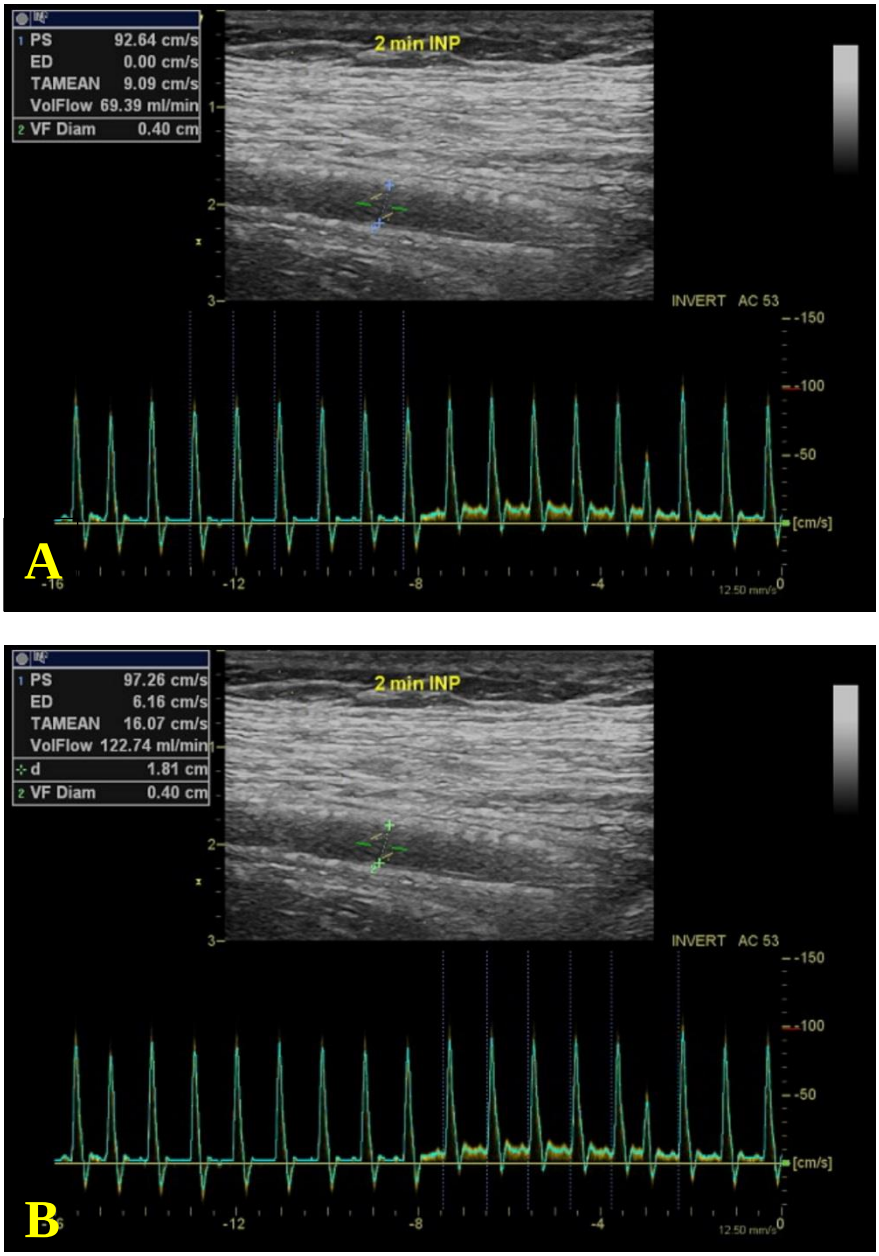

Figure 16. Volumetric flow in a stent in the superficial femoral artery.

Volumetric stent blood flow (Color flow duplex imaging) in the superficial femoral artery measured during six heartbeats before the onset of negative pressure. The average volumetric flow measured in six heartbeats at baseline (atmospheric pressure) was 69.4 ml/min. Panel B: Volumetric flow in the superficial femoral artery recorded during six heartbeats with negative pressure. The average volumetric flow measured in six heartbeats during INP (alternating 10 s with -40 mmHg and 7 s atmospheric pressure) was 122.74 ml/min. PS, peak systolic velocity; ED, end-diastolic velocity; time-averaged mean velocity, TAMEAN; VolumeFlow, the average volumetric flow during the measured heartbeats.

What this study adds and the new findings

The investigation of acute effects of INP on blood flow in patients' stents were included in this thesis to evaluate blood flow in arteries while controlling for diameter changes. Compared to atmospheric pressure within the INP cycles, INP induced increased flow and runoff in the stents in the superficial femoral arteries by 44-96 %.

How this study may impact clinical practice in the future

Endovascular treatment has become a frequent alternative to bypass surgery [270, 271]. Nevertheless, restenosis occurs in a high percentage of patients who have received endovascular treatment, especially for stents located distal to the common iliac artery [42, 272]. Several factors impact patency rate, including renal failure, plaque morphology, and antiplatelet drugs [273]. However, one of the main factors contributing to improved patency rate is runoff distal to the stent area [273]. Our results in six PAD patients with stents (nonpublished data) show that INP increases runoff through the stents and inflow to the distal part of the extremities. The clinical impact of the INP-induced blood flow needs to be evaluated in future clinical trials.

5 GENERAL DISCUSSION

Our main findings are presented and discussed in Papers I-V of this thesis. This section contains further elaboration and interpretation of these findings. The discussion in this thesis is divided into three parts. In the first part (5.1), the main results of Paper I-V are discussed. The second part (5.2) elaborates on the papers' methodological considerations and limitations, and summarizes the studies' internal and external validity. In the third part, the potential mechanisms of action and the overall relevance of the results is discussed (5.3).

5.1 Discussion of the main results

In the experimental studies (Paper I, III, and IV), we studied the acute effects of applying mild (-40 mmHg) ambient negative pressure to the lower leg and foot. The main findings of these experiments were that INP resulted in an abrupt and immediate increase in arterial and skin blood flow of the foot compared to baseline flow. Further, the increased arterial and skin blood were observed without any clinically significant changes in central hemodynamics (HR and MAP). The application of short pulses of negative pressure (mild suction) on the skin and limb increases peak blood flow in the distal foot artery and the skin during the first heart beats without changes in mean arterial pressure. This indicates that short negative pressure pulses of 10 s are safe. It also suggests that INP may be an effective means to increase microcirculation in patients with poor peripheral circulation. We observed increased blood flow pulsatility during INP compared to baseline (Paper III and IV). Additionally, in healthy volunteers (Paper I) and in PAD patients (III), application of INP resulted in increased average blood flow velocity during INP compared to baseline flow. In paper IV in people with SCI, we also analyzed the relationship between ABPI in the test foot and the difference in average blood flow velocity between baseline and the INP sequences. We found that lower ABPIs were associated with a significantly higher flow increments during INP. The reason for this relationship is unknown. The finding may, however, indicate greater flow potential in patients with poor circulation. Gill et al. [274] reported a similar relationship in patients with PAD who were exposed to constant negative external thigh pressure. Gill et al. [274] observed that the gain in toe pressure tended to be greater in PAD patients with lower pre-treatment systolic toe pressures ($r=0.52$, $P>0.05$).

Moreover, we performed additional tests on patients with stents in the superficial femoral arteries to evaluate blood flow responses to INP in fixed diameter arteries. The result was an

increase in blood flow of 44-96 % during negative pressure compared to atmospheric pressure within the INP-cycles. As the stent has a fixed diameter, this finding demonstrates that INP induced an increase in blood flow in a superficial femoral artery without any change in diameter. In a case study on patients with severe PAD and chronic leg and foot ulcers (Paper II), the main findings suggest that eight weeks of INP 2 hours per day improves ulcer healing and foot circulation. In the last paper (Paper V), we performed a clinical pilot (feasibility) study on SCI patients with chronic lower leg and foot ulcers. In this pilot study, we concluded that the portable INP device (FlowOx; Otvio AS, Oslo, Norway) is feasible for home use, and that patients were able to adhere to the prescribed intervention protocol of about two hours of INP per day for eight weeks. We also explored potential clinical effects of INP on the SCI patients. Results on the patients' ulcer indicate that adding INP to SWC improves ulcer healing compared to SWC alone. However, due to lack of statistical power, we cannot draw any finite conclusions regarding INP's effects on ulcer healing in paper V.

To the best of our knowledge, the experimental studies presented in this thesis are the first reports to thoroughly describe the isolated effects of INP applied to the lower leg and foot on arterial and skin blood flow. Additionally, we are the first to study the effects of INP therapy on ulcer healing and the feasibility using an RCT design. Our findings show that INP using FlowOx™ is a feasible method to use in a home setting, and that the patients' adherence to the treatment protocol was fairly good, with patients performing most of the 2-hour daily protocol for the eight week study time (Paper V). Taken together, the results in the experimental studies (Papers I, III, and IV) support our hypothesis that INP increases macro- and microcirculation of the foot. The clinical study (Paper V) also may indicate that INP+SWC treatment may have an additive effect on ulcer healing compared to SWC treatment alone. Our analysis in the treatment effect in Paper V, indicate that adding INP to SWC would induce an extra 42 % on ulcer healing. However, the confidence intervals were skewed due to large uncertainty in our dataset. The reason for the large confidence intervals ranging from -168 % to 73 %, is that the ulcer can never improve more than 100 % but the ulcer may be indefinite worse. Thus, 73 % means that approximately ¾ of the ulcer may be decreased after INP is added to the treatment. Large sample size would have provided smaller and more symmetric confidence intervals. Our observations in Paper V may be used to further improve the INP method and plan large-scale RCTs. In paper V, one of the patients acquired an infection in the first period, later diagnosed as erysipelas with staphylococcus aureus. This patient's symptoms got worse after 178 min use of the INP device in the second period, and

treatment was immediately discontinued. It may therefore be important for future research to test for skin infections prior to patient inclusion.

We observed increased volumetric flow during negative pressure cycles compared to cycles of no pressure during application of INP in all six of the PAD patients with stents. The increased arterial blood flow was mainly seen in diastole. Since the stent diameter is "fixed", the observed increase in flow velocity must be due to increase in blood flow. As in-stent restenosis is a major clinical problem [102, 275], improving blood flow and runoff in the stent area may theoretically assist in keeping the vessels open and thus reduce the restenosis rate. Several studies have shown increased shear stress and flow to be associated with improved graft patency [276-278], and inhibition of the development of neointimal hyperplasia [279, 280]. The INP-induced shear stress and clinical utilities in large vessels with stents need to be investigated further.

The findings in this thesis add to ample anecdotal evidence from research studies from the early 19th [164-166, 169] and 20th [171, 172, 174, 179, 180, 182, 185] centuries, from the late 1960s [37, 186], and from Scandinavian RCTs in the 1990s [187, 188]. Most of these early studies applied a combined negative and positive pressure modality, and were limited by their sample sizes and measurement techniques. Previously indirect measurements of flow such as changes in skin temperature and skin color of the lower limb have been used [171]. Our findings confirm these initial findings that application of short pulses of subatmospheric pressure results in abrupt and significant increase in blood flow.

Adequate supply of oxygen to all tissues is essential for cardiovascular health, tissue regeneration, cell proliferation, and infection resistance [281]. Patients with insufficient lower extremity tissue perfusion and non-healing (chronic) ulcers represent a large and growing group of patients with high costs to society [144]. Chronic ulcers are rarely prevalent in the healthy physiology, but more commonly affect individuals suffering comorbidities, such as diabetes, obestity, peripheral arterial disease (PAD), and neurological disorders, such as SCI [138, 144, 153]. Our clinical results on patients with lower leg and foot ulcers are encouraging, and suggest that INP enhances healing when added to SWC. Nevertheless, although our two studies indicate beneficial clinical effects on ulcer healing, the clinical benefits remain unclear. Nevertheless, the INP device in the present thesis is a promising adjunct therapy to increase peripheral circulation and improve wound healing. Because INP is pain-free, the

findings in this thesis may have implications for the treatment of peripheral arterial disease in hospitals, as well as in outpatient and community care settings.

5.2 General methodological considerations

5.2.2 Design and data analysis

Papers I, III and Paper IV were conducted using experimental designs to describe the acute effects of INP on foot circulation in the lower limbs. Due to the controlled laboratory experiments, we were able to isolate the effects of INP on foot circulation.

In Paper II, we employed a case study on PAD patients with chronic leg and foot ulcers. Although the probability to detect serious complications is low, this study design has high sensitivity in detecting novel and unexpected adverse and beneficial effects from new treatments [282]. Although case reports are considered the weakest level of evidence, they are frequently the first line of inquiry [283].

In Paper V, we performed a pilot RCT with a crossover design. RCT is considered the gold standard design for causal inference to test treatment efficacy [284]. In contrast to a parallel study — where two groups of treatment are given so the two group receives either treatment A or treatment B — in a crossover study, each patient serves as his or her own control. The crossover design reduces confounding covariates (i.e. variables which could affect the outcome of the study) because imbalances between different treatment groups are reduced. However, a confounding covariate may be plausible if the covariates change systematically during the course of the study, for example, if there were a carry-over effect from the INP (period 1) to the wound care only period (period 2) and vice versa. This could interfere with healing time efficiency in the latter stage of the study. We did not employ a wash-out phase in our study (Paper V). Because of the possibility that period 1 could interfere with the subsequent period in our crossover study design, and in order to evaluate the effect of INP on ulcer healing, we only evaluated the effect of ulcer healing in period 1. Period 2 was utilized to evaluate feasibility of the INP treatment as all patients were able to use the INP for eight weeks. In Paper II, the result showed improved wound healing of the leg and foot ulcers together with increased ABPI. From our findings in Paper II, it is therefore plausible that there was a carry-over effect after INP therapy to period 2 (SWC alone) among the SCI patients.

The crossover design is known to be statistically more efficient, so fewer participants are needed than for a parallel design. Although we planned to recruit 13 patients in each treatment group, we were unable to recruit more than nine patients by the end of December 2016. Due to the low sample size, our RCT has low statistical power and a reduced chance of detecting a true effect. Given the low sample size, the results therefore need to be interpreted with caution.

Statistical power is the probability that the study is able to detect an effect when there is a detectable treatment effect. A type I error occurs when the sample data show a treatment effect when in fact there is none (reject a true null hypothesis and accept a false alternative hypothesis). In contrast, type II error occurs when the sample does not appear to have had a treatment effect, when in fact, it does have an effect (accept a false null hypothesis and fail to accept the alternative hypothesis) [285]. To make type I error less likely, we have applied a strict level of significance (5 %). Due to the low sample size in Paper V, our results should be interpreted with caution.

We analyzed data on the basis of treatment assignment (*intention to treat principle*). In this analysis, patients are analyzed in the treatment groups in which they were analyzed regardless of what happens after randomization; regardless of the patients adherence with the treatment, regardless of the actual treatment received, and regardless of protocol deviations or withdrawal from study protocol [286]. Intention-to-treat is a conservative approach and results from such analysis have a tendency to shift toward the null hypothesis, reducing the chances of type I error (detecting an effect of INP on ulcer healing that is not presented). Nonetheless, the fact that all patients allocated to INP + SWC improved their WSA and PWAT scores after period 1 is encouraging. The feasibility results were also very promising, as adherence to the INP device was 90 % median compliance with the 2-hour protocol. Lastly, Paper V was a single-center pilot study with one of the objectives to collect additional information about the INP device, sample size, trial management and logistics before large-scale clinical studies.

5.2.3 Selection of study population

5.2.4 Internal and external validity

Internal validity is defined as the ability of a study to measure what it is set out to measure [287]. Interval validity refers to the reliability or accuracy of the results [288]. One important question

is to what extent the conclusions from the present studies can be extrapolated to other populations. External validity is the degree to which the results can be generalized to populations or groups that did not participate in the study, and is associated with the inclusion and exclusion criteria [288].

We evaluated the isolated effects of negative pressure through experimental laboratory studies. Lott et al. [289] found that increasingly higher negative pressures on the upper and lower extremities (-25, -50, -75, -100 mmHg) induce progressively larger increases in initial blood flow in both the brachial and femoral arteries. Caro [290] has also reported similar findings. Using higher negative pressure levels than -40 mmHg may therefore yield different results on both acute blood flow and clinical effects than those found in the present thesis.

In Paper I, we found that INP pulse patterns of both 15 s-15 s, 30 s-30 s and 10 s-7 s (using -40 mmHg) induced similar peak increments in blood flow, but different frequencies of flow pulsatility. We do not know whether INP patterns using high peak flow velocity induced by applying >-40 mmHg with low pulse repetitions or lower peak flow velocity with high negative pulse repetitions are most clinically efficient. That is, which pulse pattern is associated with better peripheral circulation and wound healing. Further investigations are therefore needed to compare different INP patterns (shorter vs. longer pulses of negative pressure) and different negative pressure levels (high vs. low).

In order to control for other confounding variables which could potentially result in bias of the outcome variables (such as additional heating or increased flow-mediated vasodilation from different increased shear stress caused by different negative pressure levels) — all experiments and clinical studies described in this thesis have been performed with -40 mmHg negative pressure and with stable ambient room temperature. Whether additional heating or higher negative pressure levels induce amplified blood flow responses or are associated with additional beneficial clinical outcomes should be the subject for further studies. Future investigations should also assess whether greater negative pressure is clinically feasible and safe.

Due to the convenience sampling method, few women were enrolled in our PAD sample in Paper III and none in Paper II. Nevertheless, the burden of PAD is equal among the sexes, if not higher among women compared to men [64, 65, 291]. There is a possibility that studies are influenced by selection bias in general when recruiting patients, with the notion that elderly

women who have a higher prevalence of PAD may be less prone to attend examinations [291]. Potential discrepancies between the sexes in the INP-induced flow responses may be a subject for further studies.

In our experimental studies, we aimed to perform the measurements on a relatively homogenous group of participants in each study in well-controlled laboratory conditions. We found a similar immediate increase in flow and flux after onset of negative pressure in all three study populations (as described in Papers I, III, and IV), which may indicate that the INP method applied can be used for a range of patinets types with poor peripheral circulation.

In order to investigate the clinical effects and feasibility of the INP method, we used a custom-made mobile device (FlowOx™, Otivio AS, Oslo) in relevant patient populations. In the pilot study in Paper V, we tested the effects in a pragmatic trial by measuring the extent of beneficial clinical effects in real clinical practice, as opposed to an explanatory trial with a rigorous control used to maximize internal validity [288]. We therefore did not apply strict criteria for ulcer size and type of ulcer etiology or age. People with SCI are a heterogeneous group and risk factors associated with the ulcers vary [267]. It is therefore possible that the patient population in Paper V mirrors the patient population seen in many practices, with all ulcer types and sizes.

The main limitation of our clinical studies, i.e. the exploratory case study (Paper II) and the randomized crossover pilot study (Paper V), was the low sample size. The small sample size in our pilot RCT study (Paper V) may result in failing to detect an effect or difference that is present, i.e. failure to reject a false null hypothesis (type II error). Looking at the trend in our data from both WSA and PWAT indicates that healing improved in the group receiving INP therapy + SWC vs. SWC alone. Whether this improvement also has practical significance cannot be answered from this thesis, but should be investigated by assessing clinical outcome variables in addition to ulcer healing, such as quality of life and physical function.

Both of the clinical studies (Paper II and V) had several limitations with regards to methodology which may threaten their validity (lack of blinding clinicians and patients). Nonetheless, our randomized crossover pilot study's main strength was the randomization, which is important to maintain internal validity [288]. Randomization reduces potential selection bias, which is important in order to isolate and quantify the effect of INP + SWC versus SWC. It is, however, possible that selection bias occurs in randomized trials even with perfect

randomization procedures. For example, this can occur in the way participants are rejected or accepted into the study or in the way interventions are assigned to the participants once they have been accepted into the study. To further reduce selection bias in Paper V, we employed allocation concealment. The randomization sequence was not concealed from the researchers or the patients when we obtained patients' consent. Patients were allocated after inclusion into the study and once written and oral consent were obtained. The randomization technique using sequentially numbered, opaque sealed envelopes (SNOSE), is recognized as the most accessible, simple, cheap, effective and straightforward method of maintaining allocation concealment and does not require the use of fancy or expensive technology [292, 293]. This random allocation does not, however, protect against other types of potential bias that might occur after allocation, as elaborated below.

One limitation to our clinical studies was the lack of a placebo control group. Although we included paraplegics and tetraplegics with a loss of sensation in their lower limbs, a double-blinded method using a sham intervention was not feasible in our study (Paper V), since the patients brought the INP device home and could therefore easily check whether it worked. We also did not mask the clinicians involved in the wound study, which may be a potential threat to the interpretation of the outcomes (ascertainment bias). We employed a blinded assessment of the ulcer photos and PWAT scores in Paper V to reduce the likelihood of ascertainment bias, which may otherwise threaten the study's (Paper V's) interval validity. Blinded assessment is one important technique to reduce potential ascertainment bias when the person assessing the outcome has knowledge of the group assignments [292]. Intra-class correlation for inter-observer variability for PWAT scores between the blinded wound nurse and the non-blinded physician performing the ulcer photos was 0.751 (95 % CI 0.44 to 0.89, $P<0.001$), F-test (test value 0). Likewise, ICC for inter-observer variability for WSA was 0.955 (95 % CI 0.90 to 0.98, $p<0.001$), F-test (test value 0) (Paper V). Thus, the ICC for inter-reliability indicated moderate to excellent agreement between the two experienced WSA and PWAT raters, which strengthens the validity of the ulcer evaluation used in Paper V.

The data in Paper V were analyzed on *intention-to treat basis*. Intention-to-treat analysis is accepted as the most valid analytic approach for prospective intervention studies because it adheres to the randomization procedure and is generally conservative [294]. In small sample size pilot studies, excluding data from patients with major protocol violations may potentially lead to bias, affecting the results in either direction [295].

From a start-up company's point of view, it was important to perform early testing in order to not only test efficacy (whether FlowOx™ has beneficial effects in an ideal situation), but also to perform more pragmatic testing on a range of patient populations to explore whether the INP treatment might have beneficial clinical effects. We performed several single-case products tests ("pilot testing") early on before conducting more rigorous testing. The single case testing demonstrated promising results on claudication patients [262] and chronic wound patients with severe lower extremity PAD (Fontaine Stage IV) (Sundby et al., 2016, unpublished results). This led us to conduct a more controlled case and pilot study (Paper II and Paper V). To maintain generalizability of our pilot study (Paper IV), we kept exclusion criteria to a minimum.

5.2.5 Flow and flux measurements

Blood flow velocities were used to describe changes in arterial blood flow in the foot in Papers I-IV. We were not able to measure the diameter of the dorsalis pedis and tibial posterior arteries within the subatmospheric pressure chamber during INP. If the vessels' diameter distal to the measurement site were to change due to vasoconstriction or vasodilation, this would affect the flow calculations. However, pulsatile diameter in small arteries has been found to be very stable [296]. We therefore believe it is reasonable to assume that the changes observed in blood flow velocity during INP reflect changes in blood flow in the foot arteries. If vessel diameter were to increase during the experiments (e.g. due to flow-mediated vasodilation from increased flow velocity or distending effect of the suction inside the subatmospheric pressure chamber), this would tend to further increase arterial blood flow during INP. It is unlikely that the INP-induced increase in flow velocity and shear stress would reduce vessel diameter. It is therefore more likely that we have underestimated, rather than overestimated blood flow velocity if the diameter changed during INP. Further, a study by Lott et al. [289] examined the effect of upper and lower limb suction at −25, −50, −75, and −100 mmHg. In that study, brachial and femoral arterias were found to be stable during the subatmospheric pressure application [289]. Similarly, blood flow measures (volumetric blood flow) in six additional PAD patients with a stent in the superficial femoral artery showed that INP applied to the lower leg and foot increases blood flow in the main femoral artery without any change in diameter (Sundby et al. 2017, unpublished results, Appendix 2). These results demonstrate that blood flow increased in the femoral arteries during INP without changes in arterial diameter. Finally, Fig. 4 in paper III demonstrates the strong correlation between acral

skin blood flow of the first toe, measured by laser Doppler technique, and arterial blood flow velocity. This further supports our presumption that the method to measure arterial foot blood flow velocity also reflects blood flow in the foot arteries.

None of the participants in our experiments had atrial fibrillation, which would have made our beat-to-beat analysis more difficult. However, some of the patients had a few extrasystoles (premature beats). Since we calculated beat-to-beat flow values, triggered by the ECGs' R-wave in each heart cycle, we obtained one value for each of the physiological parameters recorded (laser flux, arterial blood flow velocity). For cases in which a patient experienced an extrasystole, one heart beat may display reduced flow. However, the subsequent heart beat after an extrasystole tends to compensate with higher flow. Since we use the average flow velocity and flux values in our calculations, the extra systole is unlikely to affect average flow velocity. Moreover, each participant was subjected to many repetitions of INP: 600 s of INP with three 17 s (10 s on/7 s off) cycles per minute, for a total of 35 cycles per patient. This amounted to a total of ~700 intermittent negative cycles for the 20 patients in our study PAD in Paper III (and similarly large datasets for the other experiments presented in Papers I to IV). Given such a large dataset, a few extrasystoles would have had an insignificant impact on our beat-to-beat analyses.

Skin temperature was measured on the dorsum of the foot, and the temperature sensor was isolated from the surrounding air with several layers of adhesive tape (Micropore Surgical Tape, 3M, MN, US). It is possible however, that the tape does not adequately isolate the temperature probe and that an increase in skin temperature could be due to increased temperature inside the "air pocket" within the substmospheric pressure chamber. We did not manage to attach an extra probe inside the subatmospheric pressure chamber to measure the temperature in the "air pocket" due to problems with leakage during INP application in the transition between the seal and probe cables. Leakage results in different pressure curves, which may give a different physiological response. Nonetheless, we observed increased peak blood flow velocity in the foot arteries and increased laser Doppler flux in the pulp of the first toes during the pressure cycles. Together, these findings suggest increased tissue perfusion of the foot during INP.

A low tissue oxygenation may result from low arterial inflow, high oxygen consumption (tissue extraction) or restricted venous outflow [297]. We did not measure tissue oxygenation together with arterial, skin blood flow and skin temperature in our experimental studies (Paper

I, III, and IV). Our findings in Paper II in one patients with critical limb ischemia, suggest that $TcPO_2$ increases together with arterial and skin blood flow during INP in ischemic limbs, compared to baseline. Since tissue oxygenation and tissue perfusion are not interchangeable, combined measurement of blood flow and tissue oxygenation should be evaluated more thoroughly in future experiments on INP.

5.3 Elaboration on possible mechanisms to the increased flow pulsatility during INP

The mechanisms by which INP increases flow pulsatility warrant consideration. The exact mechanisms contributing to the increase in flow pulsatility during INP are unknown. We did not aim to examine the specific mechanisms of action in the present PhD project, and the studies were therefore not designed to do so. The section below is therefore theoretical, and somewhat speculative, based on previous findings [37, 290] and how these relate to the observations described in Papers I-IV.

The observed increase in blood flow velocity and skin blood flow during INP may be explained by increase in local perfusion pressure, or suspension or avoidance of the local venoarteriolar reflex, which constricts the arterioles when the veins become dilated. It is also possible that local vasoconstriction to some degree is affected by the intrinsic myogenic response, which is elicited when arteriolar transmural pressure increases [298]. The proposed mechanisms of action during INP are further elaborated on below.

The theoretical foundation for applying negative pressure intermittently and not continuously relates to a local vasoconstrictor response (i.e. venoarteriolar reflex) [189], which normally reduces arterial inflow when the veins overextend [196, 197, 299-302]. This deduction is based on previous work showing that constant negative pressure applied to a body part causes venous distension and reduces blood flow locally via the venoarteriolar reflex [196]. Increases in venous vascular transmural pressure of ~25 mmHg in healthy subjects have been shown to reduce local blood flow in human subcutaneous tissue [303], human cutaneous tissue [304], and human skeletal muscle [305]. This local vasoconstrictor response observed during venous stasis has been shown to be unchanged during spinal sympathetic blockade [305]. The vasoconstrictor response is, however, abolished following a local nervous blockade involving adrenergic nerves (low dose lidocaine or phentolamine not affecting myogenic activity of the smooth musculature in the vasculature), indicating that the vasoconstrictor response is due to a local venoarteriolar axon reflex [196, 303]. Stranden [193] showed that one minute of constant negative

pressure (-75 mmHg) applied to the lower body via a subatmospheric pressure chamber reduced femoral artery blood flow by approximately 50 % in healthy individuals. For patients with PAD, by contrast, the average blood flow velocity during constant negative pressure was no different from that at rest (no pressure). Further, in three patients with critical limb ischemia, blood flow increased slightly during constant negative pressure, consistent with the finding that these patients often find relief when lowering their limbs [193]. These above results suggest that the venoarteriolar reflex may play at least a partial role during negative pressure. Although there seems to be a slightly different response to constant negative pressure between atherosclerotic vessels and the vessels of healthy subjects, both the study by Stranden on PAD patients [193], and studies of healthy volunteers [289, 290] demonstrate that subatmospheric pressure applied to an extremity results in abrupt initial increases in blood flow velocity, followed by a gradual decrease towards and below baseline (resting) values.

Oxygen is transported mainly through bulk flow of blood to the capillaries (i.e. movement of molecules from an area of high to low pressure). Therefore, increased flow rates (Q) increase the delivery of oxygen via the blood to the tissues [306]. Based upon Darcy's law, Q is proportional to the pressure gradient and inversely proportional to the vascular resistance, $Q = P_1\text{-}P_2 / R$, where P_1 is inflow pressure, P_2 is outflow pressure, and R is resistance. Accordingly, flow may be increased by altering ΔP, vascular resistance, or a combination of these two factors. In the experiments in Papers I-IV, we did not observe large increases in mean arterial blood flow velocity, despite increases in flow fluctuations (pulsatility) during INP application. In Paper III on PAD patients, mean arterial flow velocity increased by 11 % while peak flow pulsatility increase by 46 % during INP application compared to the baseline sequence (no pressure). Similarly, the mean increase in arterial blood flow velocity in healthy volunteers was 8 % above baseline values, while mean pulsatility increased 44 % above baseline (Paper 1). The fact that the INP sequence induced increased flow pulsatility, but only a small increase in mean arterial blood flow, is described by the flow-response curve, where flow, after the initial increase in peak flow after about three to four seconds, gradually decreased towards and below baseline (Figure 3 Paper I; Figure 4 Paper III; and Figure 3 Paper IV). This initial increase in flow pulsatility during INP may be partly explained by temporary pressure differences within the vessels as follows:

Applying Darcy's law of flow ($Q = \Delta P/R$ or $Q = K*(P_1\text{-}P_2)$, since K (conductance) is reciprocal of R), the flow from the artery to the capillary is proportional to the pressure

gradient $Q = K*(P_a - P_{cap})$, where K is a proportional constant which depends on friction, vessel radius, vessel length, and blood viscosity. Vascular resistance, in turn, depends on the blood viscosity (which again depends on blood composition, temperature, and pressure), fluid velocity, flow regime (laminar vs. turbulent), blood vessel diameter, the number of blood vessels in parallel (network topology) and the vessels' friction parameter (e.g. atherosclerotic vessels may have higher friction than normal vessels due to more irregular shaped walls, even if the average internal diameter is equal). If capillary pressure (Pcap) suddenly decreases when applying negative pressure pulses, flow would abruptly increase due to the increased pressure gradient (as a first approximation, we neglect any change in flow from the vein side due to one-way valves in the veins) (Figure 17). We are aware that reduced pressure in the capillaries will reduce flow towards the veins, thus intermittently increasing the volume of blood stored in the capillaries (resulting in dilation and increased pressure within the capillaries). This will later enhance flow from the capillaries towards the veins immediately after the negative pressure is turned off, $Q = K*(P_{cap} - P_v)$.

We expect that after the initial increase in flow (time zero, when negative pressure is turned on), blood flow from the artery will gradually decrease towards baseline flow (no pressure), since the intravascular pressure also increases in the capillary due to continuously artery to capillary inflow, thereby reducing the pressure difference between the artery and the capillary. This theoretical model is supported by the Smyth 1969 study [37], finding that pressure in the veins and the deep calf tissue closely tracked pressure in the subatmospheric pressure chamber. Similar findings were also reported by Caro et al. 1968 [290]. By applying short pulses of negative pressure, we would expect to elicit brief periods of increased flow due to this abrupt increase in pressure gradient. This theoretical model also corresponds with other findings by Caro et al. [290] that, in healthy subjects, the application of abrupt higher negative pressures caused higher initial responses in flow compared to more negative pressures. This abrupt increase in blood flow during application of negative pressure on the lower extremities has also been observed in PAD patients [193]. Additional support for the hypothesis is given by a recent study demonstrating that increasingly more negative pressures (-25, -50, -75, -100 mmHg) induce progressively greater increases in initial arterial blood flow in both the brachial and femoral arteries during application of negative pressure on the upper and lower extremities, respectively [289].

This theory corresponds to our findings in paper I, III, and IV that blood flow velocity initially abruptly increased ~30-50 % relative to start of negative pressure (time zero), and that peak flow velocity was reached about three to four seconds after onset of negative pressure (Figure 3 in Paper I; Figure 4 in Paper III; and Figure 3 in Paper IV). After peak arterial blood flow velocities were reached, the blood flow velocities gradually decreased towards and below baseline flow (no pressure), even though the negative pressure was still on for ten seconds (Figure 3 Paper I; Figure 4 Paper III; and Figure 3 Paper IV). Because of the gradual pressure equalization (reduced pressure difference) between the artery and venous side, flow would be expected to decline gradually towards baseline values. Therefore, by applying short pulses of negative pressure, we would expect to elicit brief periods of increased blood flow due to this abrupt increase in pressure gradient.

In this theoretical "flow model" (Figure 17), we would expect an initial increase in flow during INP without taking into account flow-mediated vasodilation or other biochemical mechanisms which may also interact to enhance flow during INP. In sum, the INP-induced ambient pressure changes may provide variable driving pressure for blood flow through the cutaneous tissue, resulting in short bursts of arterial blood flow (pulsatility) to the small vessels of the skin. This, in turn, is likely to increase the flow of oxygen and nutrients to the cells, thereby improving tissue perfusion and facilitating ulcer healing. Future investigations should elucidate the exact mechanism for the observed increase in flow pulsatility during negative pressure application.

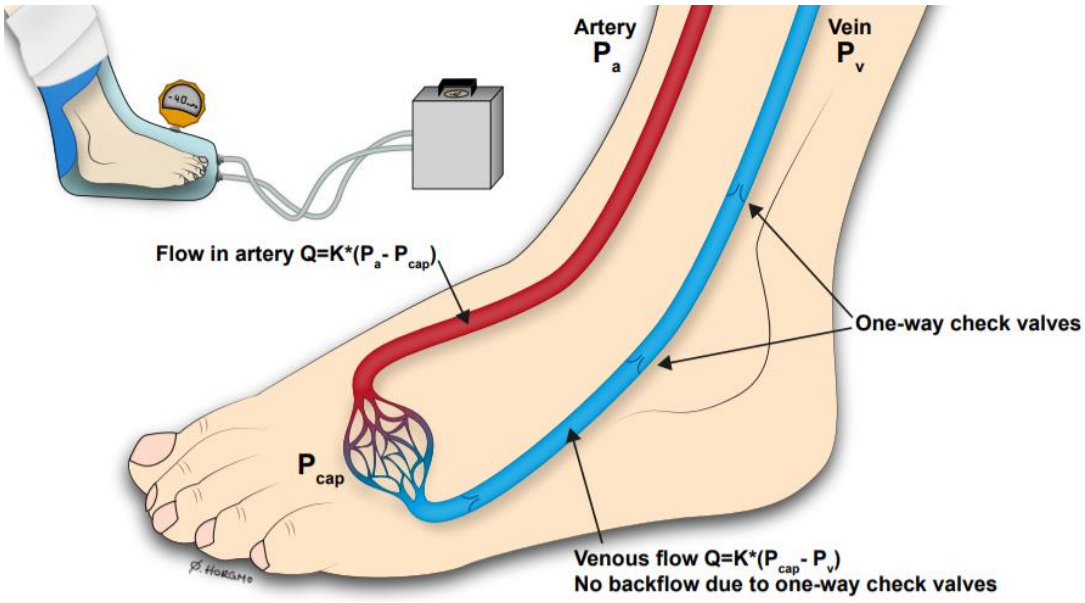

Figure 17. Schematic presentation of potential mechanism of action for the increases in flow pulsatility during intermittent negative pressure.
P_{cap} shows capillaries in close proximity to the skin with initial pressure close to atmospheric pressure (P_{atm}). Flow (Q) between the capillaries and the arteries is approximately proportional to the pressure difference $Q = K * (P_a - P_{cap})$, where Q is the flow and P is pressure, K is a proportional constant dependent on the vessel's radius, vessel length, blood viscosity and friction. If P_{cap} suddenly decreases, flow would abruptly increase (only from the artery to the capillary since veins have one-way (check) valves that prevent backflow). After this, flow would be expected to gradually decline towards baseline, since the pressure difference gradually diminishes due to increased arterial inflow (see text for further elaboration). Illustration: Øystein H. Horgmo, University of Oslo.

5.3.1 Potential biochemical effects of INP application

In the PAD patients in Paper III (Figure III, paper III), there was a significantly higher mean arterial blood flow velocity during the INP sequence compared to the 5-min baseline sequence, and this increase was sustained during the 5-min post-INP sequence (when INP was turned off). Interestingly, the increase in flow was sustained post-INP despite no increase in cumulative blood flow velocity fluctuations compared to the baseline sequence (Figure 13). This may imply that INP results in a release of vasodilatory substances secondary to augmented flow velocity. A more detailed discussion on the flow-mediated effects is outside the scope of this thesis, but can be found elsewhere [120, 307]. The INP-induced fluctuations in blood flow may spur the release of nitric oxide, resulting in improved endothelial modulation [289], activation of dormant collateral blood vessels, and potentially enabling the formation and utilization of new blood vessels.

Increased blood flow-associated shear stress is also linked to improved graft patency [277, 308]. It has been postulated that the lower shear rates (a surrogate marker for shear stress) found in the superficial femoral artery compared to the arm [309] partially explain greater susceptibility to atherosclerosis in the arteries of the leg [44]. Flow-induced shear stress from INP use may therefore explain some of the positive clinical outcomes reported in the earlier studies by Herrmann and Reid [176], Smyth [37], and our own observations in Paper II [310].

In Paper I, we found that both INP cycles of 15 s-15 s, 30 s-30 s and 10 s-7 s induce similar increments in blood flow pulsatility. More research is needed to establish the exact amount of mechanical shear and strain imposed on the vascular endothelium during different cycles of INP.

5.4 Ethical considerations and safety

The safety issues related to the use of INP are discussed at length in the methods section. Prior to the studies, ethical approval was obtained from the Regional Ethics Committee, as outlined in the respective papers (I-V). All personnel and staff involved in data collection were bound by an oath of confidentiality. All data used for analyses were made anonymous and handled according to standard regulations for clinical data. The studies involved in this project were carried out according to *The Declaration of Helsinki*. Written and informed consent was obtained from all participants at the beginning of the studies. The research participants were told that they were free to withdraw at any time without penalty.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

6.1 Main conclusions

We have in this doctoral research project demonstrated that applying short periods of negative pressure (-40 mmHg) to the lower leg and foot increases flow pulsatility to the foot. Arterial blood flow of the foot increased transiently at onset of negative pressure by about 30 % to 80 % in the small and large foot arteries, respectively, with observed increases in microcirculation of about 20 % to 89 % above resting (baseline) blood flow. Additionally, a pilot randomized controlled study indicated that INP+ SWC improved healing of chronic leg and foot ulcers compared to SWC alone. The INP method delivers negative pressure oscillation in which short pulses (10 s) of mild (-40 mmHg) negative pressure separated by shorter periods (7 s) of atmospheric pressure are applied to the lower extremities for 1-2 hours per day. Such pressure pulses did not induce clinically significant changes in central hemodynamics or unpleasant sensations.

This thesis adds novel data about the acute and long-term effects of applying INP to the lower limb on peripheral circulation and ulcer healing. Papers I, II and IV demonstrated INP-induced increases in flow pulsatility in foot macro- (Doppler ultrasound) and microcirculation (laser Doppler flux), with only minor changes in central hemodynamics (mean arterial pressure and heart rate). These results were observed in healthy volunteers, in patients with PAD, and in individuals with chronic SCI, respectively.

The studies' beat-to-beat analyses demonstrate an immediate and significant effect on peripheral macro- and microcirculation the first 2-4 s after the onset of INP. Our study on 20 PAD patients (Fontaine stage II) showed that INP induced significant increases in mean arterial blood flow (~11 %) in patients with ischemic limbs (Fontaine stage II), and that this increase in mean flow was sustained at least 5-min after INP was turned off. The cumulative change "fluctuations" in arterial blood flow velocities did not change during post-INP compared to the baseline. This suggests that INP induces physiological effects that cause a transient change within the vessels. This may have occurred through the release of local factors that cause a vasodilation response. Our randomized, controlled crossover pilot study on patients with SCI and chronic ulcers suggests that INP has an additive effect on wound healing when combined with SWC. Lastly, additional tests on PAD patients with stents in the

superficial femoral arteries showed an increase in blood flow and runoff in all six patients' stents by a range of 44-96 % (unpublished data, see section 4.12).

6.2 Specific conclusions

Regarding the specific research objectives, the conclusions are as follows:

1. In healthy volunteers, the application of mild short oscillations of intermittent negative pressure (INP) to the lower leg and foot induced increased macro- and microcirculatory blood flow pulsatility in the foot, without clinical significant changes in central hemodynamics. Furthermore, constant mild negative pressure decreased macro- and microcirculation of the foot.

2. INP induced an acute increase in blood flow pulsatility of the foot in patients with lower extremity PAD and in people with SCI. In average, INP induced a significantly increase in arterial blood flow pulsatility by a range of 33 % to 46 %, and skin blood flow pulsatility by a range of 11 % to 89 % in the patient populations tested. In patients with intermittent claudication, a small, significant increase in average blood flow velocity was also observed during both the INP and post-INP sequences compared to the baseline sequence.

3. Results from an exploratory case study on four patients with PAD and hard-to-heal leg and foot ulcers suggest that adding INP to SWC during an eight-week period improves foot perfusion and wound healing.

4. In the clinical pilot study ulcer healing improved in all of the patients during INP+SWC treatment group compared to SWC treatment alone, but the results were not statistically significant in this small pilot study. WSA improvement was seen in 4 of 4 patients for INP+SWC treatment vs. 3 of 5 patients for SWC treatment alone, while PWAT improvement was seen in 4 of 4 patients for INP+SWC treatment vs. 2 of 5 patients for SWC treatment alone. We conclude that INP applied to the lower leg and foot may have an additive effect on ulcer healing when combined with SWC compared to SWC alone in patients with SCI and chronic leg and foot ulcers of mixed ulcer etiology. However, due to slow recruitment it was not possible to reach sample size with sufficient power to answer if INP influences ulcer healing. In order to evaluate long-term effects and whether the INP method is superior to SWC alone in

inducing complete epithelization of chronic leg and foot ulcers, larger, preferably multi-center, prospective RCTs with a longer follow-up period should be considered.

5. The INP method is a feasible method to be used at home two hours per day for eight weeks for patients with SCI and lower limb ulcers.

6.3 Future research perspectives

First, the prospective clinical studies in this thesis are based on a small sample size. Further RCTs — preferably using randomized parallel group study designs — are warranted to advance knowledge about INP's potential clinical effects on patient symptoms, hemodynamics and wound healing of different ulcer etiologies.

Second, costs related to wound care are not insignificant. Wound care contributes to a substantial economic burden on health care systems, and dramatically impairs quality of life [311] and physical function [152] in the affected individuals. The true costs to the health care systems remain unknown as there is a lack of research on this topic providing costs on the hospital and community health-care providers [148]. Further, research on wound epidemiology typically consider patients with only one type of wound etiology. Thus, research providing data on the total number of patients receiving treatment related to wound care in a population is scarce [148]. The socioeconomic and individual costs are likely to increase in the years to come due to increased prevalence of lifestyle disease such as diabetes and obesity, which also follows from demographic shifts towards aging populations [312]. Despite the large and growing costs of treatment, there is a paucity of evidence-based and cost-effective treatment strategies for wound care. Cost-benefit analyses (including cost effectiveness, cost utility, and cost benefit) comparing alternative treatment therapies within a specific disorder should be carried out to compare the potential societal cost savings of integrating INP. Future studies should also include specific calculation of the economic comparison to standard of care to evaluate the direct and indirect costs associated with INP use.

Third, multi-center studies including a variety of patient populations from diabetics, to SCI to PAD would further advance knowledge about the use of INP on different ulcer etiologies. In addition, studies should also investigate outcome variables other than wound healing in order to assess long-term clinical benefits (e.g. long-term circulatory effects and vascular

endothelial specific inflammatory markers and their effect on an individual's functional outcome measures and quality of life).

Fourth, several studies found increased shear stress and flow to be associated with improved graft patency [276-278] and inhibition of the development of neointimal hyperplasia [279, 280]. The potential INP-induced increase in shear stress and potential clinical beneficial effects on patency rates in large vessels with stents should be investigated further.

Finally, well-designed studies are warranted to enhance the physiological understanding of working mechanisms that may potentially help to develop and optimize the INP method. For example, it is reasonable to assume that alternating positive and negative pressure may further enhance peripheral blood flow due to increased venous emptying (outflow) caused by the intermittent compression, as this will cause a fall in volume (after-drop) in the vein. Also applying local heating to the limb in combination with INP is likely to maximize flow to the cutaneous tissue, since it is likely to maximize cutaneous vascular conductance [313-315]. Therefore, heating should be investigated in combination with INP and compared to INP alone, as the former combination is likely to improve the impact of INP on blood flow. Studies should also explore the acute effects of different negative pressure amplitudes on blood flow and flow pulsatility. Whether INP also has an effect by suspending peripheral sympathetic regulation (veno-arteriolar response), as proposed by Rein et al. [189], remains to be examined.

6.4 Other likely clinical applications for INP

The INP induced increase in flow may also have potential clinical effects when combined with heated or cooled air or fluid to change body core temperature. For example, INP could be used during surgery (in the operational theatre) to avoid hypothermia or in military operations in warm climates to avoid hyperthermia. This type of application has been previously reported using a similar method applied to the upper extremity [189, 190].

Bone blood flow may affect bone regeneration after injury[316]. Especially in individuals with inadequate vascular function, this may induce slower recovery after musculoskeletal injuries. Another potential clinical application of the observed increase in macro- and microvascular blood flow pulsatility may therefore be to enhance musculoskeletal recovery.

7 DISCLOSURES

The present PhD project was supported in part by Otivio AS. ØHS has been a PhD student at the University of Oslo, Faculty of Clinical Medicine, and is also employed by Otivio. IM is the Chief Scientific Officer (CSO), co-founder and a shareholder of Otivio AS. IM and ØHS own shares and have options in Otivio AS. Otivio AS developed and is responsible for commercialization of the FlowOx (INP) system used in the present thesis. IM, ØHS and JH have submitted a patent application (pending) for the use of INP technology in a related field of application to the research addressed in this PhD thesis: Post-surgical occlusion treatment recovery and rehabilitation therapy. Otivio AS owns several issued patents, and IM is an inventor on multiple patent applications related to FlowOx.

Otivio AS received a research grant from The Research Council of Norway to fund an industrial PhD project, which in part funds this PhD project, including salary for ØHS (NFR grant number: 241589). Other than providing support in the form of salaries for authors IM and ØHS, Otivio AS, did not have any additional role in the study designs, data collection and analyses, decisions to publish, or preparation of manuscripts. None of the other co-authors had any personal conflicts of interest – financial or otherwise – during this doctoral project.

8 APPENDICES

Appendix 1. Contributors to the papers

Study design and planning	Paper I	Øyvind H. Sundby, Jonny Hisdal, Lars Øivind Høiseth, Jørgen. J.Jørgensen, Iacob Mathiesen
	Paper II	Øyvind H. Sundby, Jonny Hisdal, Lars Øivind Høiseth, Iacob Mathiesen
	Paper III	Øyvind H. Sundby, Lars Øivind Høiseth, Jonny Hisdal, Iacob Mathiesen, Jørgen J Jørgensen, Jon O. Sundhagen
	Paper IV	Øyvind H. Sundby, Lars Øivind Høiseth, Jonny Hisdal, Harald Weedon-Fekjær
	Paper V	Øyvind H. Sundby, Ingebjørg Irgens, Lars Øivind Høiseth, Jonny Hisdal, Harald Weedon-Fekjær
Data collection	Paper I	Øyvind H. Sundby
	Paper II	Øyvind H. Sundby
	Paper III	Øyvind H. Sundby
	Paper IV	Øyvind H. Sundby,
	Paper V	Øyvind H. Sundby, Ingebjørg Irgens, Hanne Haugland, Eivind Lundgaard
Data curation and analysis	Paper I	Øyvind H. Sundby, Lars Øivind Høiseth, Jonny Hisdal
	Paper II	Øyvind H. Sundby, Lars Øivind Høiseth
	Paper III	Øyvind H. Sundby, Lars Øivind Høiseth, Jonny Hisdal
	Paper IV	Øyvind H. Sundby, Lars Øivind Høiseth

	Paper V	Øyvind H. Sundby, Ingebjørg Irgens
Statistical analysis	Paper I	Lars Øivind Høiseth, Harald Weedon-Fekjær, Jonny Hisdal
	Paper II	N/A
	Paper III	Lars Øivind Høiseth, Harald Weedon-Fekjær, Øyvind H. Sundby
	Paper IV	Lars Øivind Høiseth, Harald Weedon-Fekjær, Øyvind H. Sundby
	Paper V	Harald Weedon-Fekjær
Illustrations and graphics	All papers	Lars Øivind Høiseth, Øyvind H. Sundby, Øystein Horgmo
Writing original draft of the manuscript	All papers	Øyvind H. Sundby
Manuscript revision and accepted final version of the manuscripts	Paper I	Øyvind H. Sundby, Jonny Hisdal Lars Øivind Høiseth, Iacob Mathiesen, Jørgen J.Jørgensen, Harald Weedon-Fekjær
	Paper II	Øyvind H. Sundby, Lars Øivind Høiseth, Jonny Hisdal, Iacob Mathiesen, Jørgen J.Jørgensen
	Paper III	Øyvind H. Sundby, Jonny Hisdal Lars Øivind Høiseth, Iacob Mathiesen, Jon O. Sundhagen, Harald Weedon-Fekjær
	Paper IV	Øyvind H. Sundby, Lars Øivind Høiseth, Jonny Hisdal, Iacob Mathiesen, Jon O. Sundhagen, Gunnar Sandbæk
	Paper V	Øyvind H. Sundby, Lars Øivind Høiseth, Ingebjørg Irgens, Jonny Hisdal, Iacob Mathiesen, Jon O. Sundhagen, Hanne Haugland, Eivind Lundgaard, Gunnar Sandbæk
Proofreading and final spell-check	All papers	Annie Bersagel

Appendix 2. Thesis at a glance

- **Paper I -** *Application of intermittent negative pressure on the lower extremity and its effect on macro- and microcirculation in the foot of healthy volunteers.*

  Aim: To determine the effect of different patterns of negative pressure on foot perfusion in healthy volunteers.

  Participants: 23 healthy volunteers.

  Methods: Experimental physiological study, randomized.

  Ethics: The *Regional Committee for Medical and Health Research Ethics* in Norway (protocol number: 2014/1967).

  Conclusions: Intermittent mild negative pressure (-40 mmHg) significantly increases fluctuation in macro- and microcirculation distally in the foot. Constant negative pressure decreases mean arterial blood flow velocity, while short oscillating intermittent mild negative pressure (-40 mmHg) increases mean arterial blood flow velocity and pulp skin blood flow in the foot.

- **Paper II –** *The effects of intermittent negative pressure on the lower extremities' peripheral circulation and wound healing in four patients with lower limb ischemia and hard-to-heal leg ulcers: a case report.*

  Aim: To explore the potential clinical effects of eight weeks of INP therapy in patients with peripheral arterial disease and hard-to-heal leg and foot ulcers.

  Participants: Four patients with peripheral arterial disease and hard-to-heal leg and foot ulcers.

  Methods: Exploratory non-randomized eight-week case study.

  Ethics: The *Regional Committee for Medical and Health Research Ethics* in Norway (protocol number: 2015/1318).

Conclusions: These cases suggest that INP may facilitate wound healing and improve foot perfusion. Well-designed, adequately powered RCT studies are needed to investigate the potential clinical effects on wound healing and foot perfusion.

- **Paper III -** *The acute effects of lower limb intermittent negative pressure on foot macro- and microcirculation in patients with peripheral arterial disease.*

  Aim: To describe the effects of applying INP to the lower leg and foot on foot macro- and microcirculation and central hemodynamics in patients with PAD.

  Participants: 20 patients with lower extremity PAD with claudication pain (Fontaine Stage II).

  Methods: Experimental study.

  Ethics: The *Regional Committee for Medical and Health Research Ethics* in Norway (protocol number: 2014/1967).

  Conclusions: INP increases flow pulsatility in foot macro- and microcirculation in patients with PAD, while also increasing mean arterial blood flow velocity. The mean arterial blood flow velocity is sustained for at least 5-min after termination of INP.

- **Paper IV -** *Intermittent negative pressure applied to the lower limb increases foot macro- and microcirculatory flow pulsatility in people with spinal cord injury.*

  Aim: To investigate the acute effects of INP on foot circulation in people with chronic spinal cord injuries (SCI).

  Participants: 24 people with SCI

  Methods: Experimental study

  Ethics: The *Regional Committee for Medical and Health Research Ethics* in Norway (protocol number: 2014/1967).

  Conclusions: INP increases blood flow pulsatility in foot macro- and microcirculation in patients with SCI.

- **Paper V -** *The effects of intermittent negative pressure applied to the lower limb on leg and foot ulcer healing in patients with spinal cord injury: a clinical crossover pilot study.*

  Aim: To explore the potential clinical benefits of repetitive use of INP on patients with spinal cord injury (SCI) and chronic leg and foot ulcers.

  Participants: 9 SCI patients with chronic leg and foot ulcers.

  Methods: Randomized controlled, assessor-blinded, crossover, pilot study.

  Ethics: The *Regional Committee for Medical and Health Research Ethics* in Norway (protocol number: 2015/1318).

  Conclusions: Seven of nine patients completed the study protocol without side effects, and adhered to about 90 % of the prescribed INP protocol. Ulcer healing was measured as change in ulcer area, using a Photographic Wound Assessment Tool. All patients in the INP+SWC treatment group improved. However, the results were not statistically significant in this small pilot trial. WSA improved in 4/4 patients for INP+SWC vs. 3/5 patients for SWC, while PWAT improved in 4/4 patients vs. 2/5 patients. Due to low sample size, the treatment effect is inconclusive.

- **Unpublished supplementary results on PAD patients' stent blood flow**

  Aim: To measure the effect of INP applied to the lower leg and foot on blood flow in large static arteries.

  Participant: Six patients with peripheral arterial disease and stents in their femoral superficial arteries.

  Methods: Experimental study.

  Ethics: The *Regional Committee for Medical and Health Research Ethics* in Norway (protocol number: 2015/1318).

  Conclusions: INP increased blood flow without changing vessel diameter in superficial femoral arteries with stents. Compared to atmospheric pressure within the INP cycles, INP induced increased blood flow in the stents in the superficial femoral arteries by a range of 44-96 %. INP using -40 mmHg negative pressure oscillations may have a positive effect

on runoff in superficial femoral artery stents. Prospective studies should investigate the potential clinical effects on patency rates in patients with poor runoff.

Appendix 3. Populærvitenskapelig sammendrag

Redusert blodtilførsel til benet kan føre til smerter, sår som ikke gror, og i verste fall amputasjon. Forekomsten av kroniske sår er høy blant pasienter med åreforkalkninger, diabetikere, eldre, og personer med ryggmargsskade. Antall pasienter med kroniske sår i verden er økende.

I avhandlingen "The effects of mild lower limb intermittent negative pressure on foot circulation and wound healing" har Øyvind Heiberg Sundby og medarbeidere studert en metode som bruker mildt pulserende undertrykk i en spesiallaget støvel på leggen og foten for å øke sirkulasjonen i benet.

Sundby og medarbeidere har vist at kontinuerlig undertrykk reduserer blodstrømmen til foten, mens pulserende undertrykk øker blodstrømmen. Metoden et testet på friske forsøkspersoner, ryggmargsskadde og hos pasienter med åreforkalkninger i beina. Studiene viser at metoden ikke medfører noe ubehag eller påvirkning på blodtrykket. Våre pilotstudier på pasienter med perifer karsykdom og ryggmargsskadde med kroniske legg og fotsår indikerer at behandling med pulserende undertrykk i kombinasjon med sårbehandling har bedre effekt enn dagens sårbehandling.

Hovedfunnene fra avhandlingen er at trykk som senkes i korte perioder gir økt blodstrøm i fotpulsåren og i huden ytterst i foten. Resultatene på sårtilheling tyder på at det er grunnlag for å studere den potensielle kliniske gevinsten av dette videre i større randomiserte kontrollert studier.

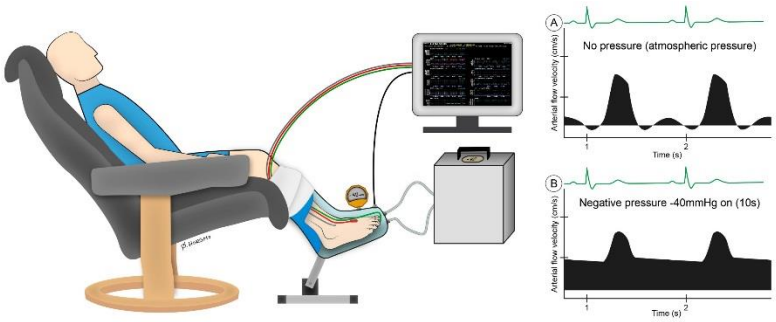

Figure 18. Popular science summary explaining the experimental set up.

Populærvitenskapelig sammendrag med forklaring av forsøksoppsettet. Figuren viser hvordan probene for måling av sirkulasjonen i foten er festet og koblet opp til en datamaskin og analyseprogramvare. Den ene foten er plassert i et luft tett kammer (FlowOx™, Otivio AS, Oslo, Norge). I tillegg er blodstrømmen, målt med ultralyd Doppler hastighetsmåler i hvile (A) og under perioder med pulset undertrykk (B). Illustrasjon: Øystein Horgmo, Universitet i Oslo.

Appendix 4. Overview and summary of key literature.

Table 4. Studies describing the application of ambient negative or alternating negative and positive pressures to an upper or lower extremity.

Reference (year)	Design	No. of participants	Participant profile	Intervention type	Measurement	Results	Main limitations
Herrmann and Reid [171, 181] (1934)	Reviews	Unknown. General review of studies employing ambient pressure to the extremities between 1812 to 1934	Claudicants, Peripheral vascular disease	Intermittent positive and negative pressure. The automatic pressure applies alternating pressures of -80 mmHg and +20 mmHg [181]. Gradual change in pressure: ~15s	Herrman and Reid reports of more than 3600 PAVEAX treatments. Increase in walking distance Improved skin vitality. Increase in skin temperature. Symptom relief.	Intermittent positive and negative pressure is reported* to benefit many patients, including pain relief and increased walking distance. The author claims that the treatment has saved amputations in many patients	No RCTs. Anecdotal description of the results. Based on multiple case reports. Lack of objective blood flow measurements. Use of self-reported walking distance
Landis and Hizrot [180] (1935)	Clinical study	n = 30	Peripheral vascular disease: Thromboangiitis Obliterans, arteriosclerosis, ulcers, diabetes	Intermittent positive and negative pressure as described by Landis and Gibbon [179], using an aluminum pressure chamber. Pressure was alternated using negative pressure of -80 mmHg to -120 mmHg (below atmospheric pressure) for 25 s and +60 mmHg to +80 mmHg for 5s with change between	Ulcer healing, claudication distance/exercise tolerance, pain, skin color	Reports improved claudication distance and exercise tolerance, skin color, ulcer healing in several patients. Of 9 diabetics with ulcers, 6 patients were described as good outcome, 1 fair and 2 did not improve. Of 5 diabetics without ulcers, 4 improved, 1 did not improve. In 9 patients with Thromboangiitis Obliterans	No RCT. Use of less objective outcome measures. Methods of measurements not well described. Outcome measures described as clinical evaluation with relief of symptoms and signs of disease.

				pressures in ~3 s. Treatment length >1-2 h/day and 3 times per week as symptoms diminished.		with ulcers, 5 had good outcome, 2 fair (some relief of symptoms and signs of disease) and 2 none. In 3 patients with Thromboangiitis Obliterans without ulcers, 2 had good ouctomes and 1 had fair outcome. In 3 patients with arteriosclerosis, 2 had good outcomes and 1 had no improvement. In 1 patients with arteriosclerosis without ulcers the patients had a good outcome.	
Allen and Brown [182] (1935)	Clinical cases from the Mayo clinic	n = 60 reported cases	Peripheral arterial disease	Alternating -60 to -80 mmHg and +20 mmHg with 4 cycles/min. Treatment given ranging from 7 days to 66 days and from 7 hours to 154 hours in total.	Wound healing, changes in pain, skin temperature	Seven patients with thrombo-arterioslcerosis obliterans and 4 with thromboangiitis obliterans were treated, and all but 2 patients pain was relieved during treatment. Two of the patients experienced increased pain during the treatment. Three patients got permanently relieved of the pain. Skin temperature was measured on 35 patients, and the temperature increased in average by 2°C after 68 treatments. Of ten with ulcers and gangrene, four patients improved healing, while 6 did not show any benefits.	No RCT, no controlled environment during measurement of skin temperature. Different treatment duration given to patients. No standardized protocol of ulcer measurement
Smyth [37] (1969)	Experimenta l and clinical study	n = 46 patients in total with peripheral	Patients with peripheral vascular disease. 40	INP applied to the whole lower limb with 15 s on (-150 mmHg) and 15 s off at	Walking distance (self-paced), skin vitality,	INP increased self-reported walking distance in the majority of the patients. After 6 weeks of INP treatment,	The population is not described in detail. No randomization or control group, small sample size

		vascular disease (PVD); n=40/46 with intermittent claudication. 3/46 had Raynaud's disease, 2/46 had postoperative ischemia following arterial reconstruction, one had angina of effort, 3/46 had Raynaud's disease and ulcers	patients with intermittent claudication, 3 with Raynaud's disease, 2 with post-operative ischemia following arterial reconstruction, 1 with angina at physical effort	atmospheric pressure for 30 min 2 times/week Also measured acute changes in flow in a few patients	skin temperature, ulcer healing, clearance of subcutaneous injected adrenaline in hands of 2 normal subjects tested with and without applying negative pressure	resting blood flow in the calf of 19 patients increased significantly by 282 % (P<0.001), and reactive hyperemia increased by 300 %. During INP in one patient, both blood flow velocity in the femoral artery and venous outflow velocity increased during negative pressure. INP appears to be useful in treatment of patients with PVD. Author reports more than 2000 cases have been tested without any adverse reactions and many of these patients also report increased walking distance. 3 patients' ulcers completely healed. Clearance of injected subcutaneous adrenaline showed skin surface was flushed at -60 mmHg, and the drug disappeared after a few seconds. Without negative pressure application, adrenaline was visible for 20-30 min	with only 3 patients with ulcers. Use of self-reported walking distance. Ultrasound Doppler measurements only in one patient and no detailed flow description
Gill et al. [186] (1974)	Presentation of paper at meeting of the Surgical Research Society 4th and 5th of	n = 15 patients,	8 = "obliterative arterial disease," 7 with Raynaud's disease of the hands + 5 controls	Protocol: 30 min INP of 15s on (-150 mmHg) and 15 s off, 3 times/week for 4 weeks, as also described by Smyth (1969).	Calf muscle blood flow.[133] XE clearance to measure skin blood flow. Toe and finger blood flow measurement	Statistically significant increase in finger and arm skin temperature after 4 weeks. Increase was maintained for at least 4 weeks. No statistically significant change in leg skin temperature or toe blood flow after treatment.	Brief report on study methods and results. No detailed blood flow data from the experiments or about the populations are provided. Lack of statistical information with P-values. Relatively small sample size. Different methods

	January 1974				s using venous occlusion plethysmography. Limb skin temperature before and after max vasodilation. Ankle systolic pressure before and after 4 treatment periods.	Resting and post-exercise calf muscle blood flow increased significantly after treatment period compared to before treatment. Increase in resting ankle systolic pressure. Control group remained unchanged.	of skin blood flow measurements on the upper and lower extremities.
Agerskov et al. [317](1990)	Experimental study	n = 9 patients (4 women, 5 males)	Median (range) age: 62 (45-77) year. Median systolic ankle pressure: 56 (range 95-28), One patient with non-insulin dependent diabetes mellitus, one patients with claudication peak claudication of <50 m, 8 patients with rest pain.	Constant negative pressure applied around thigh (-35 to -45 mmHg) using an airtight box. Negative pressure applied for 10-15 min. Measurements before, during, and after negative pressure application.	Toe blood pressure and skin blood flow in first toes (133Xe wash-out method) Central hemodynamics (HR, MAP), serum protein and hematocrit.	Median (range) systolic toe pressure increased from 32 mmHg (range 5-70) at baseline (before experiment) to 44 mmHg (range 10-88) after experiment (P<0.05), Toe blood pressure tended to be greater the lower the pre-test toe pressure was, correlation coefficient r = 0.52 (P>0.05). Relative skin blood flow measured in first toe increased by 304 % (range 86-767) (*P*<0.05), during INP. No change in heart rate, systemic blood pressure, skin temperature, serum protein and hematocrit during each phase.	Small sample size. Lack of control group, sham intervention, and randomization at baseline. Did not measure flow in the leg not exposed to INP.

						During constant negative pressure, rest pain relieved in 6 of 8 cases and aggravated in 1 patient. The patient with claudication felt no change. Author concluded that -35 to -45 mmHg constant negative pressure around thigh increases foot perfusion, probably due to change in collateral arterial resistance in the thigh.	
Himmelstrup [187] et al. (1991)	Controlled, randomized double-blinded, crossover study	n = 22 patients	Patients with stable intermittent claudication. Median (range) age: 65 years (54-77) and median duration of intermittent claudication of 5 years were randomized to either 25 active or 25 placebo treatments over 2 months. Measurements then repeated and placebo group received 25 active treatments over a period of 2 months followed by registration of	Combined negative and positive pressure using a semicollasible treatment system (Vacusac Intl.aps, Denmark): Boots of hard felt and blanket of porous felt wrapped around body from top of the boots to the level of axillae, fastened around the hips. Airtight plastic bag drawn up from below and firmly secured with adhesive tape around chest. The bags contain valves connected to electronically negative pressure pump to yield oscillating negative pressure of 30% and amplitude of 5% of the atmospheric pressure lasting 5-10s. Treatment duration: 15 min. Placebo treatments: Plastic bags with negative pressure for 30s, and then only atmospheric pressure. Total of 25 active treatments. Measurements	Systemic and peripheral systolic blood pressures. Treadmill pain-free and maximal walking distance. Ankle pressure index (ankle systolic pressure/arm systolic pressure) and toe pressure index (toe systolic pressure/arm systolic pressure).	17 patients completed study. The active treatment significant increased pain-free walking distance from 54m (24-107 m) to 99 m (30-420 m) (P< 0.05) and increased maximal walking distance from 99 m (36-182 m) to 185 m (68-591 m) (P<0.05). No change in either walking outcomes in placebo treated group (pain-free walking distance: 44 m (18-74 m) to 51 m (14-100 m) (*P*>0.05); maximal walking distance: 69 m (58-130 m) to 98 m (40-199 m) (*P*>0.05). In crossover, the placebo group received active treatment over 2 months: Pain-free walking distance increased from 51m (14-100 m) to 86 m (18-1000 m) (P<0.05) and maximal walking distance from 98m (40-199 m) to 175m (51-1000 m) (*P*<0.05). Patients randomized to active	Relatively small sample size. Lack of detailed information about the study populations' medical history.

			the various variables.	made by same two nurses, who were unaware of patients' randomization.		treatment group increased ankle pressure index from 0.46 (0.39-0-67) to 0.58 (0.46-0.94 (P<0.05), while the toe pressure index remained unchanged. No change in placebo group's ABPI and toe pressure indices. Systolic blood pressure decreased in the active treatment group, remained unchanged in the placebo group. 6 months' reassessment showed pain-free and peak walking distances still increased in both groups (P<0.05). Before start of treatment, 12 out of 17 patients reported sensation of cold feet. After active treatment, 2 out of 17 patients reported sensation of cold feet.	
Mehlsen et al. [188] (1993)	Double-blinded, randomized trial	n = 34 patients (12 females, 22 males)	Median (range) age: 67(54-78). Median disease duration: 4.5 years (1-15) years. All claudicants with ankle-pressure indices: 0.34-0.83.	Combined negative and positive pressure using a semicollasible treatment system (Vacusac Intl.aps, Denmark). Compared effects of 25 Vacusac treatments to 25 placebo applications given over period of 2 months. Patients randomized to either 25 active treatments or 25 placebo treatments (n = 22) or 25 active	Pain-free and maximal walking treadmill distance, ADP threshold for platelet aggression, fibrinolytic activity from euglobin	Pain-free walking distance increased significantly from 54 m (24-107) to 99 m (30-420) in active group. No changes in placebo group [(44 m (18-74) to 51 m (14-100)]. Peak walking distance increased significantly in active group from 99 m (36-182) to 185 m (68-1000). No change in placebo group: 69m [(58-130) to 98 (40-	No report of ADP threshold for platelet aggression and fibrinolytic activity in placebo group. Not designed to reveal whether changes due to positive or negative pressure alone

				treatments (n = 12) over 2 months with the equipment comparing the effects on platelet aggregation and fibrinolysis. Treatment duration: 15 min. Oscillating negative pressure of -228 mmHg.	clotylisis time.	199)]. Ankle-pressure index increased significantly in active treatment group from 0.46 [(0.39-0.67) to 0.58 (0.46-0.94)] in active treatment group. Increases due to decreased systolic blood pressure of upper limbs since pressure was unchanged in the ankle. ADP threshold for platelet aggression increased significantly in active treatment group. Fibrinolytic activity tended to increase in the active treatment group.	
Rein et al. [189] (2007)	Randomized experimental study	n = 22	Patients undergoing prolonged laparotomy for gastric surgery.	Treatment 1: Heated water (42.5°C) + INP alternating 10s -40 mmHg and 7 s atmospheric pressure applied in cylinder on one arm. Treatment 2: Forced-air warming (43°C) (Bair Hugger®) on thoracic and upper arm surface.	Body core temperature, systolic and diastolic pressure, skin blood flow and skin temperature in contralateral arm (control).	Two methods performed similarly during first 60 min, with mean 0.78°C decrease in core temperature. Core temperature curve in INP group then increased and returned to baseline (37°C) by 120 min. Temperature of forced-air heating group increased more slowly, reaching 36°C by 120 min (P<0.05).	The study did not measure arterial blood flow or skin blood flow in treated extremity.
Rein et al. [190] (2014)	Randomized experime	n = 9	Healthy subjects	The study compared effects of two devices on healthy volunteers under continuous passive heat stress in a climatic chamber. Subjects	Body core temperature	Core temperature continued to rise during treatment with constant negative pressure device, whereas it dropped slightly and then stabilized	No measurement of blood flow or skin temperature in treated arm.

	ntal study	exposed to ambient temperature above skin temperature and high air humidity randomized to either constant negative pressure (-40 mmHg) and cooling temperature of 19°C (CoreControl®) or INP (-40 mmHg) with cooling water (19°C) (Thermotube).	during treatment with INP device.

Appendix 5. Errata

The following approved changes have been made from the original version submitted to the committee:

1. Inserted page “updates” in PREFACE with updated link to published articles (Paper IV and V).
2. p. 124: Added the number 9 to the headline; “REFERENCES” is corrected to “9 REFERENCES”.
3. In the Table of Contents for Appendices, Appendix 5 p. 107: “Papers I-V” is corrected to “Papers I-V p. 157”
4. A space has been added before and after the following symbols throughout the thesis to make the text consistent:
   a. percentage (%) signs
   b. equal (=) signs
   c. after s (seconds)
5. Corrected spelling mistakes or edits to make the text consistent:
   p. 20: Reference 12 changed to font 12: (…) “arteries [12]” is corrected to “arteries [12]”
   p. 22: “endothelium” is corrected to “endothelium”
   p. 22: “aanalogous” is corrected to “analogous”
   p. 40: “129,248 USD” is corrected to “US$ 129,248”
   p. 101: “respective papers (I-IV)” is corrected to “respective papers (I-V)”
   p. 102: The number “80” is moved to the start of the next line
   p. 107: Contributors to papers “p. 104” is corrected to “p. 108”
   p. 107: “Thesis at a glance p. 106” is corrected to “Thesis at a glance p. 110”
   p. 107: Popular science summary [Populærvitenskapelig sammendrag] “p. 110” corrected to “p. 114”
   p. 107: Overview and summary of key literature “p. 111” is corrected to (…) “p. 115”
   p. 115: “2 5s” is merged and corrected to “25 s”

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10 PAPERS I-V

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ORIGINAL RESEARCH

Application of intermittent negative pressure on the lower extremity and its effect on macro- and microcirculation in the foot of healthy volunteers

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Arterial blood flow velocity, dorsal pedis artery, intermittent negative pressure, laser doppler fluxmetry, skin blood flow.

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Abstract

Intermittent negative pressure (INP) applied to the lower leg and foot may increase peripheral circulation. However, it is not clear how different patterns of INP affect macro- and microcirculation in the foot. The aim of this study was therefore to determine the effect of different patterns of negative pressure on foot perfusion in healthy volunteers. We hypothesized that short periods with INP would elicit an increase in foot perfusion compared to no negative pressure. In 23 healthy volunteers, we continuously recorded blood flow velocity in a distal foot artery, skin blood flow, heart rate, and blood pressure during application of different patterns of negative pressure (−40 mmHg) to the lower leg. Each participant had their right leg inside an airtight chamber connected to an INP generator. After a baseline period at atmospheric pressure, we applied four different 120 sec sequences with either constant negative pressure or different INP patterns, in a randomized order. The results showed corresponding fluctuations in blood flow velocity and skin blood flow throughout the INP sequences. Blood flow velocity reached a maximum at 4 sec after the onset of negative pressure (average 44% increase above baseline, $P < 0.001$). Skin blood flow and skin temperature increased during all INP sequences ($P < 0.001$). During constant negative pressure, average blood flow velocity, skin blood flow, and skin temperature decreased ($P < 0.001$). In conclusion, we observed increased foot perfusion in healthy volunteers after the application of INP on the lower limb.

Introduction

The idea that air pressure can be manipulated to increase peripheral circulation was first described in the mid-19th century (Murray 1832, 1835; Junod 1833; Clanny 1835). Expanding upon this discovery, Sinkowitz and Gottlieb (1917) published the first account of the application of a "suction-device" to treat patients with peripheral arterial disease (PAD) in 1917. The device used constant negative pressure to improve peripheral circulation of the lower

limbs. Similarly, several investigations published in the 1930s on the use of intermittent negative and positive pressure on patients with PAD obtained promising results. The studies demonstrated increased skin temperature and wound healing in the foot, as well as limb salvage and improved pain management (Herrmann and Reid 1934; Shipley and Yeager 1934; Takáts 1934; Landis and Hitzrot 1935; Conway 1936).

A previous investigation has demonstrated a large increase in vascular resistance and a subsequent fall in blood flow during application of constant negative pressure (Skagen and Henriksen 1983). Increased venous pressure elicits a vasoconstrictor reflex and substantially reduces blood flow in cutaneous, subcutaneous, and skeletal tissues (Henriksen 1976; Henriksen and Sejrsen 1976, 1977; Skagen and Bonde-Petersen 1982; Skagen and Henriksen 1983).

In the late 1960s, Caro et al. (1968) demonstrated that a rapid application of negative pressure on the forearm resulted in an abrupt and immediate increase in blood flow in the brachial artery. Smyth (1969) found a large and repetitive increase in arterial inflow in the calf and the femoral arteries when applying short oscillations of intermittent negative pressure (INP) to the lower extremities in individuals with peripheral vascular disease. These results suggest that peripheral blood flow can be increased by means of INP with short oscillations of negative pressure applied directly to a limited part of the lower extremity. Recently, Rein et al. (2007) suggested that applying INP using 10 sec of −40 mmHg and 7 sec of atmospheric pressure increased blood flow to the arm. Rein et al. (2007) suggested that by applying negative pressure intermittently, the vasoconstrictor effect of the venoarterial reflex may be circumvented (Rein et al. 2007). Thereby, short negative pressure oscillations may facilitate an acute and repeated increase in arterial and cutaneous blood flow. However, the effects of INP on macro- and microcirculation in the foot remain poorly understood.

The aim of the present experimental study was therefore to determine the effects of different patterns of negative pressure on foot perfusion in healthy volunteers. The study was designed to examine the acute hemodynamic changes during four different sequences of negative pressure applied to a lower extremity. On the basis of previous reports, we hypothesized that short periods with INP would elicit an increase in arterial and cutaneous blood flow in the foot compared to no negative pressure.

Materials and Methods

Participants

We recruited 25 healthy volunteers after obtaining their written informed consent: 15 men and 10 women. Inclusion criteria were: (1) good general health and fitness with no abnormal cardiovascular findings on clinical examination, (2) no history of drug abuse (including alcohol), and (3) no medication during the course of the study or past 30 days preceding the study. The participants were normotensive (blood pressure < 140/90 mmHg), nontobacco users, and were free of any known cardiovascular, metabolic, or neurological diseases.

The participants were asked to refrain from eating 2 h before the start of the study and from consuming alcohol or caffeine 24 h before the start of study. They were also asked not to take any vitamin supplements 72 h before study or to perform intense physical activity 12 hours before their visit. All participants had a normal ankle-brachial index (ABI) (≥1.0), pulse volume recordings (PVR) waveforms with amplitude >10 mm, and a brisk upstroke and downstroke with dicrotic notch, indicating good peripheral circulation. The participants were informed about the general nature of the experiment and the length of each sequence, but not the order of interventions. The experimental protocol was approved by the Regional Committees for Medical and Health Research Ethics in Norway (protocol number: 2014/1967) and performed in accordance with the Declaration of Helsinki.

Experimental design

The participants were comfortably clothed and seated in an armchair for 30 min before the experiment started. After 2-min baseline registrations with no pressure manipulation (atmospheric pressure, 760 mmHg), four sequential INP cycles with −40 mmHg (compared to atmospheric pressure) were applied for 2-min in a randomized order: (1) 2-min constant negative pressure, (2) 30 sec negative pressure/30 sec atmospheric pressure, (3) 15 sec negative pressure/15 sec atmospheric pressure, or (4) 10 sec negative pressure/7 sec atmospheric pressure (Fig. 1). Randomization was performed by generating random numbers in Microsoft Excel (Microsoft Office 2010 for Windows; Microsoft, Redmond, WA). After a wash-out period of minimum 5 min, the same protocol was repeated, giving participants two sets of measurements of all five 2-min sequences. Randomization limited the order effect and a 5 min wash-out period between sets reduced any potential carry-over effect. All investigations were conducted in a quiet, temperature-controlled environment (24.6 ± 1.5°C) to reduce sympathetic stress that could create artifacts (Thoresen and Walloe 1980).

Anthropometric measurements

After taking measurements of stature (Modell MZ 10023, ADE, Hamburg, Germany) and body weight (Seca medical

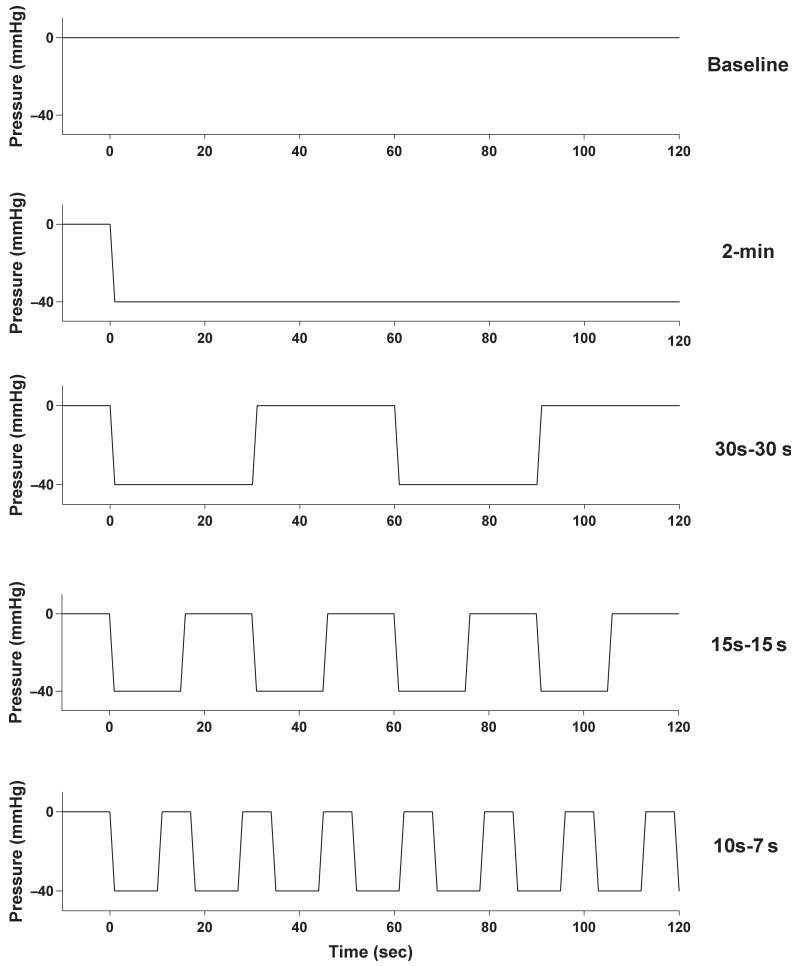

Figure 1. Illustration of the five 2-min sequences. From top: Baseline, 2-min constant negative pressure, 30 sec-30 sec, 15 sec-15 sec, and 10 sec-7 sec. All negative pressure oscillations: −40 mmHg.

scales, Seca 877, Hamburg, Germany), we obtained ABI measurements with the participant in a supine position, using a continuous-wave hand-held 8 MHz Doppler blood velocity detector (Macrolab, STR Teknikk, Aalesund, Norway). We used a blood pressure cuff to measure systolic blood pressure to the nearest 2 mmHg in the brachial artery of both arms. For the legs, we used a Doppler detector to measure systolic blood pressure to the nearest 2 mmHg in the dorsalis pedis artery. We calculated the ABI numerator by dividing the higher ankle systolic pressure (obtained from the dorsalis pedis arteries) by the higher of the two arm systolic pressures (Aboyans et al. 2012). Last, we took pulse-volume recordings (MacroLab, STR Teknikk, Aalesund, Norway) in the lower limbs by means of an air-plethysmography cuff placed on each participant's distal ankle (Bo et al. 2013).

Signal acquisition and analysis

We measured arterial blood flow velocity ($cm \cdot sec^{-1}$) in the dorsalis pedis/posterior tibial artery with a 10 MHz pulsed Doppler probe (SD-50; GE Vingmed Ultrasound, Horten, Norway). The ultrasound beam was positioned centrally at either the dorsum pedis or posterior to the medial malleolus of the ankle, depending on where we obtained the highest velocity signal. We did not measure the diameter in the dorsal pedis/tibialis posterior artery, but previous research has shown that the pulsatile diameter in small arteries is very small (Eriksen 1992). We therefore assumed that the changes in observed arterial blood flow velocity during negative pressure oscillations reflect changes in blood flow in the foot artery and not only changes in the artery diameter.

We used laser Doppler fluxmetry (LDF; Periflux PF 4000; Perimed AB, Järfälla, Sweden) to measure acral skin blood flow, giving a semi-quantitative real-time measurement of cutaneous peripheral microcirculation expressed in arbitrary units (AU) (Sarnik et al. 2007). After preparing the skin with an alcohol swab, we attached LDF probes (404-1; Perimed AB, Järfälla, Sweden) bilaterally to the skin of the pulps of the big toes.

We recorded finger arterial pressure continuously from the third finger of the right hand using a photoplethysmographic volume-clamp method (Finometer; FMS Finapres Medical Systems BV, Amsterdam, The Netherlands). At the beginning of each study, Finapres pressures were calibrated with measurements from an automated sphygmomanometer connected to a patient monitor (Solar 8000i; GE-Marquette Medical Systems, Inc., Milwaukee, WI). Skin temperature was continuously measured within the boot on the foot, in close proximity to the dorsalis pedis artery, using an Analog Devices AD590 temperature transducer (STR Teknikk, strteknikk.no, Aalesund, Norway).

Analog signals for all measurements were sampled at 300 Hz and averaged for each heartbeat gated by the R-waves of the 3-lead ECG using custom-made software (REGIST 3; Morten Eriksen, University of Oslo, Oslo, Norway). In all experiments, measurements were carried out by the same researcher. The participants were instructed to avoid moving and talking throughout the sampling period.

Application of intermittent negative pressure

During the test runs, the participants were asked to sit comfortably in an armchair with an approximate angle of 130° in their knee and hip joints (Fig. 2). The participants' body and legs were covered with a blanket. After attachment of probes, both of the participants' feet were covered with loose, non-elastic wool socks to keep their feet warm and under thermoneutral conditions during the course of the study. The right leg was placed in a rigid molded polyethylene boot coupled to a pressure control system (FlowOx™; Otivio AS, Oslo, Norway), with the contralateral leg acting as a control. The boot had internal padding to allow insertion of a leg with probes and prevent pressure points on the leg and skin. The boot was sealed just below the knee with a thermoplastic elastomer (TPS-SEBS) to allow for application of negative pressure. Pressure was continuously monitored throughout the study using a calibrated pressure transducer (Fluke, 700G Series, Everett, WA) attached to the boot and analyzed in REGIST 3 (Fig. 2).

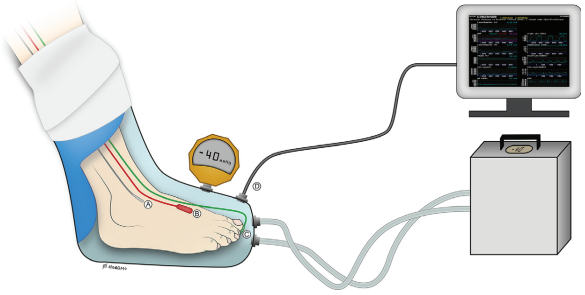

Figure 2. Illustration of the test setup with probes attached to the foot. The participant's leg was placed in a custom-made chamber interfaced with the pressure control system. The chamber acted as a vacuum chamber, by sealing around the participant's leg below the knee. The left leg was placed outside the vacuum chamber, acting as a control in atmospheric pressure. (A) Skin temperature probe; (B) Ultrasound Doppler probe; (C) Laser Doppler flux probe; (D) Pressure transducer from boot interfaced with the computer. An additional external pressure device was connected to the boot in order to calibrate boot pressure. Illustration: Øystein H. Horgmo, University of Oslo.

Tolerability of negative pressure

At the end of each experiment, the user was asked to rate their level of comfort during INP using a verbal numerical pain rating scale 0–10, ranging from no pain or discomfort (0) to worst imaginable pain (10).

Statistical analysis

All data are presented as mean (95% CI) unless otherwise stated. Hemodynamic measures were captured in a baseline sequence without manipulation of pressure prior to each randomization. We repeated all sequences, except the baseline, in a randomized order. Effects of the four different sequences over time compared to baseline were evaluated in a regression model, by assigning variables to each of the different sequences. Due to data correlation within participants, we analyzed the data in a linear mixed regression model with participants as a random effect to account for individual differences, using the R nlme package (R Foundation for Statistical Computing, Vienna, Austria). The intercept of the regression model gave the estimate of flow in the baseline sequence. The effects of each variable gave the differences from the baseline sequence. Confidence intervals for the different sequences analyzed in the mixed regression model were extracted using the "glht" function of the "multcomp" package in R (R Foundation for Statistical Computing, Vienna, Austria).

To estimate the maximal effect of INP on blood flow, we aggregated the first 10 sec of all the negative pressure oscillations in both sequences. To avoid using the same data to both locate the maximal blood flow velocity and estimate

its magnitude, we used the first set to locate the time of maximal blood flow velocity and the second set to estimate its magnitude, and vice versa. For the 10 sec-7 sec, 15 sec-15 sec, and 30 sec-30 sec-seqences, we evaluated maximum blood flow velocity using only the first negative pressure period within each sequence. To evaluate the effects of pressure over time within each sequence, we rounded the time of each observation (heartbeat) to the nearest second. Variables were assigned to each second, and effects of pressure over time within sequences were evaluated and compared to the values from the first second of each sequence. For all statistical tests, a two-tailed probability level of $P < 0.05$ was considered statistically significant.

Results

Twenty-five healthy volunteers (15 males, 10 females) were included in the study. Due to small movements during suction, adequate, high-quality Doppler signals could not be obtained from two participants. Consequently, the results of 23 participants were available for analysis (15 males, 8 females). Table 1 presents the 23 participants' anthropometric data. On the verbal numeric rating scale (0–10), one participant rated discomfort at 1 while the rest rated 0 (Table 1).

Blood flow velocity in the foot over whole 2-min registration period

Median baseline arterial blood flow velocity captured during a 2-min registration period was 8.9 (6.4–11.4) cm•sec^{-1}. Compared to the 2-min baseline sequence (atmospheric pressure), blood flow velocity was increased in the 10 sec-7 sec and 30 sec-30 sec sequences to 9.6 (7.1–12.1) and 10.1 (7.6–12.6 cm•sec^{-1}), respectively (both $P < 0.001$). Blood flow velocity did not significantly change during the 15 sec-15 sec sequence: 8.8 (6.3–11.2) cm•sec^{-1} ($P = 0.24$). A reduction in blood flow velocity compared to the 2-min baseline was observed in the sequence with 2-min constant negative pressure: 7.8 (5.3–10.2) cm•sec^{-1} ($P < 0.001$). Beat-by-beat plots of the median blood flow velocities are presented in Figure 3.

Peak blood flow velocity occurred after about 4 sec with an increase of 44 (55–33) % from the onset of negative pressure ($P < 0.001$) (Fig. 4). The blood flow velocity response to INP varied between and within each participant (range: ~40–200% increase in blood flow velocity). See Figure 5 for a representative blood flow velocity response to INP in one participant.

Table 1. Demographic and anthropometric data, $n = 23$[3].

Variables[1]	
Age (year)	27 (7.6)
Body mass (kg)	73.5 (11.8)
Stature (cm)	179 (0.1)
BMI (kg•m^{-2})	22.8 (2.0)
Ankle-brachial index (%)	107.0 (0.1)
PVR right (mm)	20.9 (9.4)
PVR left (mm)	18.3 (7.2)
Systolic blood pressure (mmHg)	113.7 (10.0)
Diastolic blood pressure (mmHg)	70.7 (6.8)
Mean arterial pressure (mmHg)	85.0 (7.1)
Verbal numeric pain scale (0–10)[2]	0.0 (0–10)
Aerobic exercise (h•week^{-1})	5.2 (4.0)
Training frequency (times•week^{-1})	5.8 (3.6)

[1]Values are mean (SD).
[2]Values are median (min-max).
[3]15 males and 10 females.
BMI, body mass index; PVR, pulse volume recording.

Laser Doppler fluxmetry and skin temperature

Effects of different sequences averaged over the entire 2-min registration period

The mean baseline flux was 74 (49–110) AU. In the right (intervention) foot, all sequences except the 2-min sequence were associated with increases in flux ($P < 0.001$), with the largest increases during the 10 sec-7 sec sequence with a mean of 90 (67–134) AU ($P < 0.001$). In the left (control) foot, all sequences were associated with a decrease in flux compared to the baseline sequence ($P < 0.001$). Flux increased simultaneously with blood flow velocity. Maximum flux was reached about 2 sec after the onset of negative pressure, with the highest values attained during the 10 sec-7 sec and 15 sec-15 sec sequences (Fig. 4).

Mean skin temperature during the 120 sec sequences was 26.4 (25.3–27.5)°C at baseline, 26.5 (25.4–27.7) °C in the 10 sec-7 sec, 26.5 (25.4–27.6)°C in the 15 sec-15 sec, 26.5 (25.4–27.7)°C in the 30 sec-30 sec, and 25.5 (24.4–26.6)°C in the 2-min sequences.

Central hemodynamics

There were only minor and no clinically significant changes in heart rate (HR) and mean arterial pressure (MAP) between the different pressure sequences. Mean HR at baseline was 62 (58–67) beats•min^{-1}. There was a difference of less than two heartbeats between baseline, INP, and constant negative pressure sequences: 10 sec-7 sec: 61.5 (61.3–61.7) beats•min^{-1}, $P < 0.001$; 15 sec–15 sec: 60.9 (60.7–61.1) beats•min^{-1} ($P < 0.001$); 30 sec-30 sec: 62.1 (61.9–62.3) beats•min^{-1} ($P = 0.42$); 2 min: 62.4 (62.2–62.6) beats•min^{-1} ($P < 0.001$).

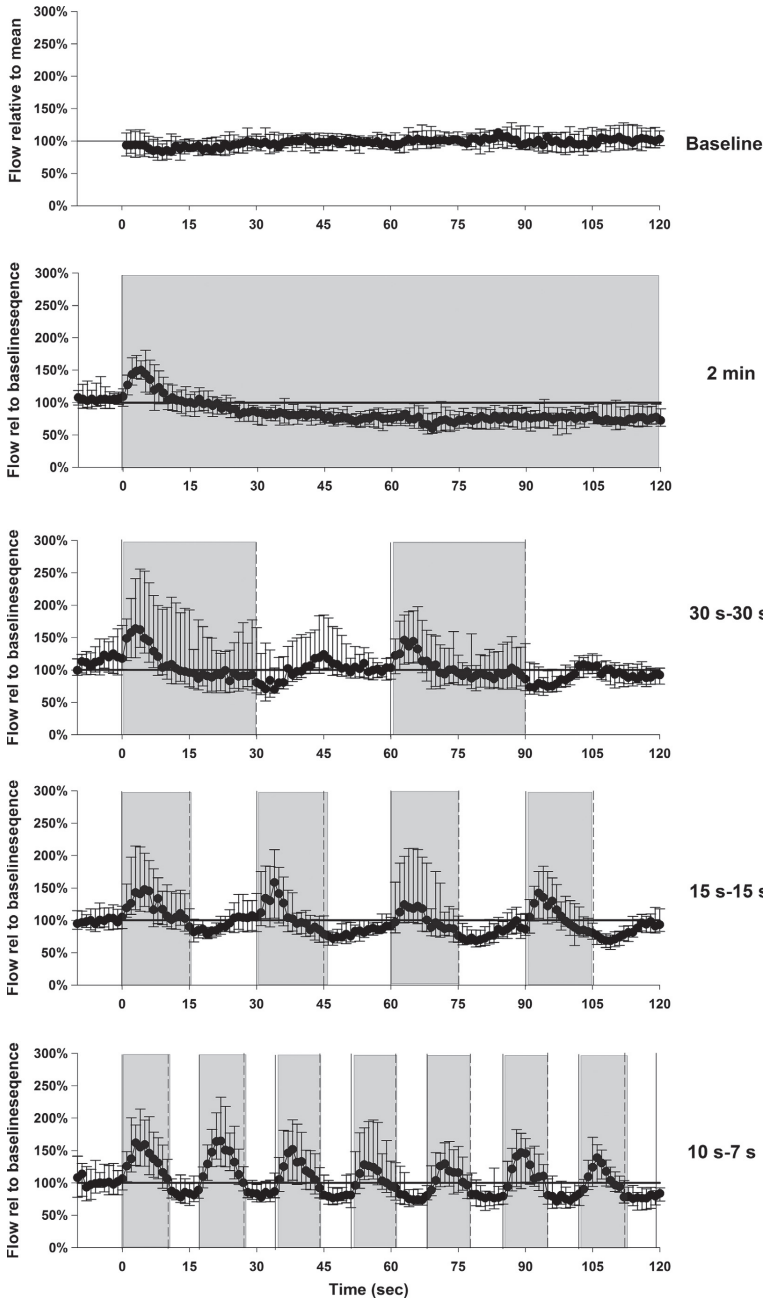

Figure 3. Blood flow velocity in the dorsal pedis artery/posterior tibial artery (ultrasound Doppler), during 2-min period in atmospheric pressure (baseline) and in four different sequences of negative pressure. The data are normalized for each subject to the average of the baseline period. Values are median and 25th and 75th percentiles. Grey color indicates negative pressure.

MAP was 67 (57–77) mmHg at baseline. During the negative pressure sequences, MAP increased by less than 2.5 mmHg: 10 sec–7 sec: 68.9 (68.7–69.1 mmHg ($P < 0.001$); 15 sec-15 sec, 69.6 (69.4–69.9) ($P < 0.001$); 30 sec–30 sec: 69.1 (68.9–69.3) mmHg ($P < 0.001$); 2 min: 69.4 (69.2–69.7) mmHg ($P < 0.001$).

Discussion

The main finding in this study was that in all sequences, arterial blood flow velocity increased after the onset of negative pressure, with peak flow after 4 sec. On average, we observed a peak increase of 44% compared to

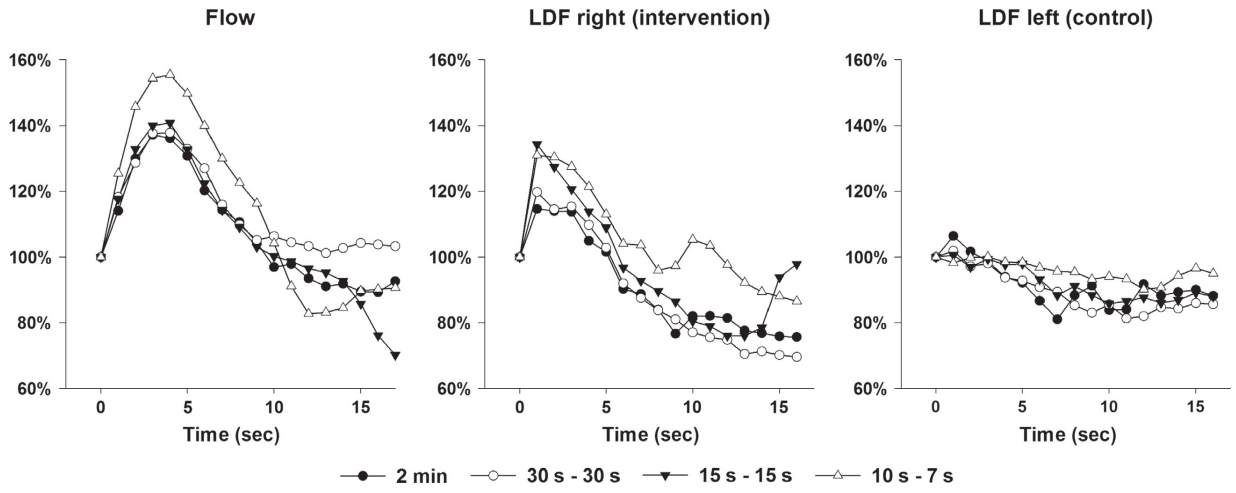

Figure 4. Effects of time after onset of negative pressure comparing different sequences. All INP-sequences were aggregated, and estimated by assigning variables to each second in a linear mixed regression model. All values are relative to the baseline sequence (100%). ''LDF right'' is measured on the foot with negative pressure, whereas "LDF left" is measured on the contralateral foot (control leg). Flow, Blood flow velocity; LDF, laser Doppler fluxmetry.

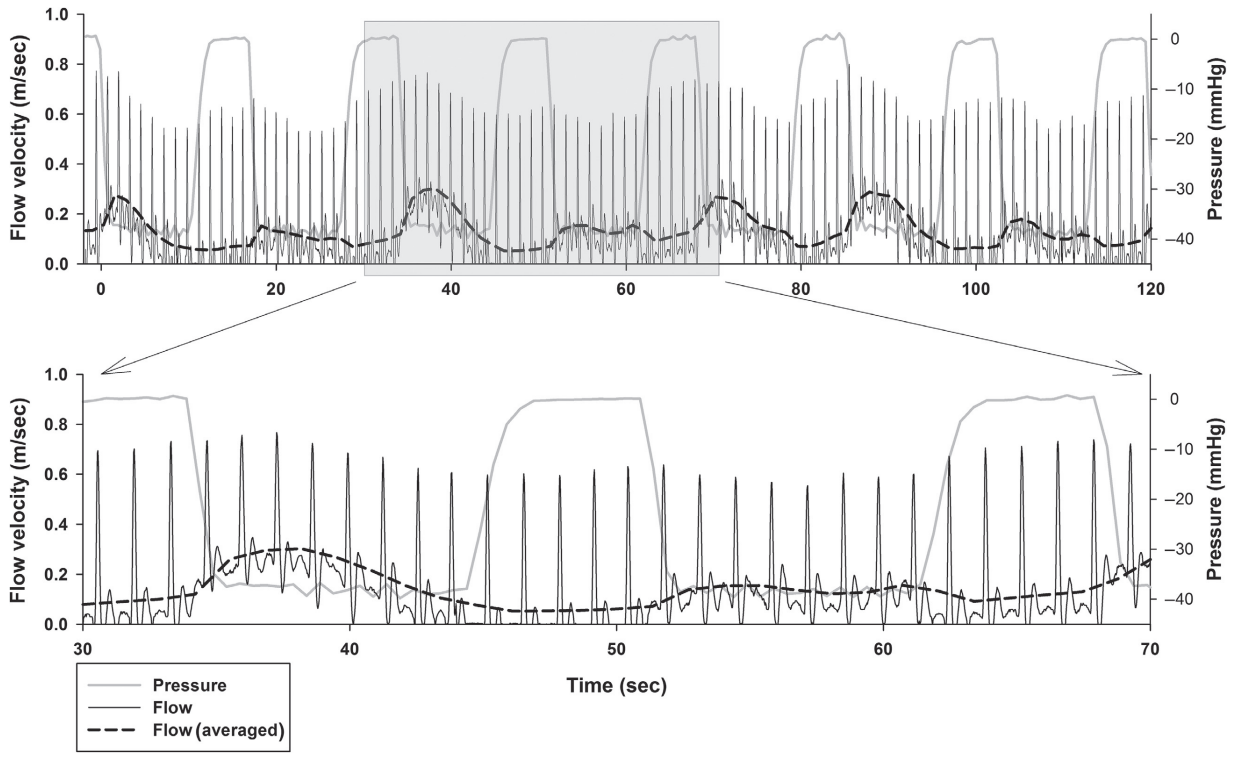

Figure 5. A representative 10 sec-7 sec sequence from one participant. Blood flow velocity per heartbeat (ultrasound Doppler) is displayed in solid black lines. The dashed lines show the trend line for average blood flow velocity per heartbeat. Both are measured along the left *y*-axis. Boot pressure grey lines, right *y*-axis. Upper panel is an entire 120 sec sequence, shaded area is zoomed in lower panel.

baseline. In addition, we observed a corresponding increase in acral skin blood flow. Last, arterial blood flow velocity, skin blood flow, and skin temperature decreased following the application of constant ambient negative pressure. The findings support our hypothesis that the application of oscillating negative pressure, applying brief periods of intermittent negative and atmospheric pressure, increases arterial blood flow velocity and skin blood flow in the foot of healthy participants.

Our results support previous reports where the same INP sequence was applied to the arms in combination with temperature-controlled water to influence body core temperature (Rein et al. 2007, 2014). In the first study, Rein et al. (2007), demonstrated that combining warm water and oscillations of 10 sec of negative pressure and 7 sec of atmospheric pressure to an upper extremity was more efficient than conventional forced-air heating in preventing hypothermia during laparotomy. In their second study (Rein et al. 2014), the authors exposed volunteers to passive heat stress while testing the effect of two portable negative pressure devices on core body temperature. The authors demonstrated significantly increased cooling efficacy using the 10 sec-7 sec INP-sequence in combination with a temperature of 24°C that covered the whole arm, compared to a device that applied constant negative pressure and cooling temperature of 19°C sealed around the hand (CoreControl, Avacore Inc. Ann Arbor, MI). Unfortunately, Rein et al. (2007, 2014) did not measure arterial lood flow velocity and acral skin in the arm exposed to INP. In this study, we observed an increase in blood flow velocity and acral skin blood flow during each negative pressure oscillation. In addition, we were also able to record a small increase in skin temperature on the dorsum of the foot during all INP sequences. Although the 2-min INP sequences applied in this study increased skin temperature while the constant negative pressure decreased skin blood flow, these changes were small and probably clinically insignificant. Interestingly, Herrmann and Reid (1934) reported increased skin temperature at rest in PAD patients treated with intermittent negative and positive pressure several hours per day for 2 weeks or more. Skin temperature is an indirect measure of improved skin blood flow, and together with the increased LDF, these observations suggest that INP improved blood flow in the small vessels of the skin.

Smyth (1969) found increased arterial inflow in the leg arteries of patients after 6 weeks' application of an INP sequence of 15 sec of negative pressure and 15 sec of atmospheric pressure. In the same study, the researchers also observed increased femoral arterial blood flow velocity during application of a 15 sec-15 sec sequence (Smyth 1969). When comparing the ultrasound measures in the calves of 11 patients with severe peripheral vascular disease before and after 6 weeks of twice-weekly INP treatment, the authors observed an increased average resting blood flow of 282% compared to pretreatment (Smyth 1969). The Smyth study also reported increased peak reactive hyperemia after occlusion by 300% compared to pretreatment. In our study, the 15 sec-15 sec sequence generated greater fluctuations in blood flow velocity than the baseline sequence, the 2-min pressure sequences, and the 30 sec-30 sec sequence (Fig. 3). The average blood flow velocity in the 15 sec-15 sec, however, was not significantly different from baseline (Fig. 3). In general, the difference in average blood flow velocity between baseline and during INP sequences (10 sec-7 sec and 30 sec-30 sec) in this study was minor, and probably not clinically significant. One possible explanation for this finding may be that the observed increase in flow during the first seconds after onset of negative pressure was counteracted by a reduction, starting 4 sec-5 sec after the onset of negative pressure (Figs. 3 and 4). It is possible that the clinical effects of 6 weeks of INP therapy on wound healing, blood flow and walking distance described by Smyth (1969) were due to the pulsatile shears stress or other biochemical effects from fluctuations in blood flow and not the average increase in blood flow.

There have been many recent studies describing the importance of arteriolar vasomotion and capillary flow motion for nutrition and oxygen supply to the tissues (Stucker et al. 1998; Li et al. 2006; Thorn et al. 2009, 2011). Although the mechanism by which this occurs is still largely unknown, flowmotion is regarded as more essential than average flow in determining adequate tissue perfusion (Stefanovska 2009). In our study, the frequent oscillations of negative pressure elicited pulsatile flow, indicated by large fluctuations in arterial and skin blood flow during the negative pressure cycles. Such fluctuations have been linked to beneficial effects in tissue perfusion. First, the dynamic rhythmic fluctuations in capillaries and arteries (flowmotion) are believed to be an important factor in tissue oxygenation (Tsai and Intaglietta 1993; Aalkjaer et al. 2011). Second, the high-flow velocity oscillations are believed to induce sufficient shear stress on the endothelium to stimulate the release of biochemical mediators. For example, increased pulsatile flow—in contrast to cells exposed to steady shear stress—has been demonstrated to increase prostacyclin (Frangos et al. 1985), endothelial-derived relaxing factor (Cooke et al. 1990), platelet-derived growth factor (Hsieh et al. 1991), and tissue-type plasminogen activator (Nollert et al. 1991) in cell culture models. Also, a randomized trial on patients with peripheral arterial disease showed that combining intermittent positive and negative pressure improved walking distance and the

adenosine diphosphate threshold for platelet aggregation (Mehlsen et al. 1993). Although care should be exercised in interpreting the results of this study, the enhanced peripheral circulation we observed with the use of INP may have clinical implications. Nevertheless, further studies are needed to elucidate any potential mechanisms and clinical implications of the pulsatile flow observed during INP.

In this study, the 2-min constant negative pressure sequence induced both reduced arterial blood flow velocity (Fig. 3) and pulp skin blood flow, which was accompanied by a small reduction in skin temperature. This reduction in arterial blood flow in a limb when exposed to constant negative pressure has been associated with the local sympathetic venoarteriolar reflex (Henriksen 1976; Skagen and Henriksen 1983).

This study demonstrated that repeated application of mild INP using −40 mmHg evoked a repetitive increase in local macro- and microcirculation with only a small, probably clinically irrelevant, increase in mean arterial pressure in healthy participants. Compared to the baseline, heart rate was only 0.5 beats•min^{-1} and 1.1 beats•min^{-1} lower during 10 sec-7 sec and 15 sec-15 sec sequences, respectively, while it increased <0.5 beats•min^{-1} for the 30 sec-30 sec and 2-min sequences. These differences were small and probably clinically insignificant. It is possible that the small increase in systemic blood pressure is caused by an increase in total peripheral resistance as a result of repeated activation of the venoarteriolar reflex at the onset of negative pressure. In this study, none of the participants reported any pain or unpleasant sensations during INP application. This was confirmed by a verbal numerical rating score close to zero. Consequently, the application of INP may facilitate repetitive arterial inflow, while at the same time avoiding the contact rash and skin abrasion often associated with compression (Moran et al. 2015).

Limitations to the study

There are some limitations to be addressed. First, we did not design this study to monitor venous pressure, and are therefore not able to elaborate about the mechanisms behind the changes in blood flow observed during application of negative pressure in the foot. Further investigations are therefore warranted to study the exact mechanisms. Second, in this study we did not measure the diameter of the dorsal pedis/tibialis posterior artery during INP. However, we found a similar response between blood flow velocity and acral skin blood flow, measured with laser Doppler flux. Eriksen (1992) found pulsatile diameter in small arteries to be very stable, and Hisdal et al. (2001) observed a strong correlation between acral skin blood flow, measured by the laser-Doppler technique, and blood flow velocity. We therefore assume that the changes observed in the blood flow velocity during INP reflect changes in blood flow in the foot artery, and not changes in artery diameter. Last, this study was conducted on healthy volunteers, and care should be exercised when extrapolating the results to different patient groups, such as those with peripheral arterial disease, diabetes or a dysfunctional autonomic nervous system, that is, following spinal cord injury.

Conclusion

This is the first study to describe the effects of INP on skin blood flow and arterial blood flow velocity. To the best of our knowledge, it is also the first to compare different sequences of negative pressure oscillations on lower limb perfusion. This study found that application of frequent mild intermittent negative pressure (INP) of −40 mmHg in the foot in healthy volunteers induced rhythmical fluctuations in blood flow and increased both arterial blood flow velocity and skin blood flow. The significance of the observed transient increase in peripheral circulation for oxygen supply and tissue proliferation requires further investigation to examine the clinical effects of repetitive use. Future investigations should also examine the working mechanisms of INP.

Acknowledgments

The authors would like to thank the volunteers for their participation in this study. We appreciate Otivio AS for procuring laboratory probes, boot, and vacuum-pump for the use in the INP study. We would also like to thank Annie Bersagel for language editing, and Øystein Horgmo at the Photographic and Video Services, University of Oslo, for providing the illustration of the test setup.

Conflict of Interest

The study was supported in part by Otivio AS. ØHS is a PhD student at University of Oslo employed by Otivio. Otivio AS owns and has the commercial rights to the INP technology used in the study. IM is the CEO, a co-founder and a shareholder of Otivio AS. None of the other authors have any personal conflicts of interest – financial or otherwise. The authors alone are responsible for the content and writing of the paper.

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II

Physiological Reports

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CASE REPORT

The effects of intermittent negative pressure on the lower extremities' peripheral circulation and wound healing in four patients with lower limb ischemia and hard-to-heal leg ulcers: a case report

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Keywords
Blood flow, intermittent negative pressure, leg ulcer, peripheral arterial disease, wound healing.

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Abstract

Peripheral circulation is severely compromised in the advanced stages of peripheral arterial disease. Recently, it was shown that the application of −40 mmHg intermittent negative pressure (INP) to the lower leg and foot enhances macro- and microcirculation in healthy volunteers. In this case report, we describe the effects of INP treatment on four patients with lower limb ischemia and hard-to-heal leg and foot ulcers. We hypothesized that INP therapy may have beneficial hemodynamic and clinical effects in the patients. Four patients (age range: 61–79 years) with hard-to-heal leg and foot ulcers (6–24 months) and ankle-brachial pressure indices of ≤0.60 on the affected side were included. They were treated with an 8-week intervention period of −40 mmHg INP (10 sec negative pressure and 7 sec atmospheric pressure) on the lower limbs. A custom-made vacuum chamber was used to apply INP to the affected lower leg and foot for 2 h per day. After 8 weeks of INP therapy, one ulcer healed completely, while the other three ulcers were almost completely healed. These cases suggest that INP may facilitate wound healing. The theoretical foundation is that INP assists wound healing by improving blood flow to the small blood vessels in the affected limb, increasing the flow of oxygen and nutrients to the cells.

Background

Arterial leg ulcers result from insufficient blood supply to the tissues. In patients with advanced stages of peripheral arterial disease (PAD), ulcers of the lower leg are common, due to reduced microcirculation in the extremities (Mekkes et al. 2003; Grey et al. 2006). Unfortunately, the treatments available for these patients are often

insufficient (Wolfe and Wyatt 1997), frequently causing complications and unnecessarily long healing times (Marston et al. 2006). Revascularization below the knee shows poor long-term results (Gray et al. 2014). A common method used to treat acute and chronic wounds is topical negative pressure wound therapy (NPWT) where a pump applies continuous or intermittently negative pressure to a sealed dressing in the wound area, removing wound exudate from the exposed area into a canister (Dumville et al. 2016). Unfortunately, the use of NPWT in the local wound environment and its impact on healing of arterial leg ulcers and tissue perfusion in clinical use are inconclusive (Wackenfors et al. 2004; Gregor et al. 2008; Vig et al. 2011; Shon et al. 2014).

Recently, we developed a method that applies intermittent mild negative pressure (INP) to the lower leg and foot in a chamber sealed below the knee (Sundby et al. 2016). In a study on healthy volunteers, we observed increased acute arterial and skin blood flow in the foot (Sundby et al. 2016). The rationale for applying INP is the finding that constant negative pressure applied to an extremity causes venous distension and reduces blood flow locally via a vasoconstrictor mechanism, the veno-arterial reflex (Skagen and Henriksen 1983). Smyth (1969) applied a similar INP methodology to the lower limb (−150 mmHg), and observed increased wound healing and walking distance after 6 weeks of INP-therapy in patients with peripheral vascular disease (Smyth 1969). Despite these results, little attention has been given to the INP method in recent years.

Four patients with ischemic limbs and hard-to-heal leg and foot ulcers underwent 8 weeks of INP-therapy (Table 1). In addition, we describe in detail two of the patients with the lowest foot perfusion at inclusion. The patients received standard wound care at the local hospital's wound clinic for 5–18 months before inclusion in this study. They agreed to take part in an 8-week pilot study on the clinical effect of a novel INP method on wound healing. The experimental protocol was approved by the regional ethics committee (REK Sør-Øst 2015/1318). Written informed consent was obtained from all patients to publish this report.

Based upon the aforementioned studies (Smyth 1969; Sundby et al. 2016), we hypothesized that INP-therapy

Table 1. Characteristics of the wound patients (n = 4) exposed to 8 weeks of INP-therapy. See text for a detailed description of patient 1 and patient 2.

Patients	Measurements	Demographic	Week 0 (Baseline) Wound leg	Week 8 (Completion) Wound leg
1	Age (year)	63.0		
	Height (cm)	176.0		
	Weight (kg)	76.0		
	Time with wound (mo)	24		
	Wound size (cm)		6.5 × 2.7	4.5 × 1.8
	ABPI		0.50	0.67
2	Age (year)	61.0		
	Height (cm)	176.0		
	Weight (kg)	76.0		
	Time with wound (mo)	24		
	Wound size (cm)		4.0 × 4.5/1.6 × 1.6	1.5 × 1.5/Epithelialized
	ABPI		0.46	0.62
3	Age (year)	74.0		
	Height (cm)	180.2		
	Weight (kg)	75.7		
	Time with wound (mo)	6		
	Wound size (cm)		0.5 × 0.5	Epithelialized
	ABPI		0.51	0.54
4	Age (year)	79.0		
	Height (cm)	178.0		
	Weight (kg)	77.6		
	Time with wound (mo)	8		
	Wound size (cm)		4.5 × 3.5	Epithelialized
	ABPI*		N/A	N/A

ABPI, Ankle-Brachial Pressure Index.
*Not applicable due to calcified vessels.

would increase macro- and microvascular capacity in the lower extremity, and that this would facilitate adequate perfusion for wound healing to occur. INP therapy was performed at home with a portable device (FlowOx™, Otivio AS, Oslo, Norway). The device consists of a vacuum chamber and a pump. The method is described in detail elsewhere (Sundby et al. 2016). The vacuum chamber is sealed around the patient's leg below the knee (Fig. 2). Negative pressure cycles are created by alternating between removing air (10 sec of −40 mmHg) and venting the chamber to atmospheric pressure (7 sec). The patients were instructed to use the INP device at home for a total of 2 h, divided into two sections of 1 h per day. Treatment compliance was observed by recording INP time on a USB memory stick provided with the device. Wound size for each patient was measured by the wound nurses prior to and after 8 weeks of INP-therapy. Macrocirculation was assessed in a supine position by the ankle brachial pressure index (ABPI) with a hand-held 8 MHz Ultrasound Doppler blood velocity detector. Additionally, a pulse volume recording (PVR) (MacroLab, STR Teknikk, Aalesund, Norway) was performed. Microcirculation was assessed by skin perfusion pressure (SPP) using SensiLase PAD-IQ (Vasamed Inc., Eden Prairie, MN).

Report

Patient 1

The first patient was a 63-year-old Caucasian male, smoker, and non-diabetic (Table 1). His medical history included myocardial infarction, a cerebral insult, smoking-induced chronic obstructive pulmonary disease, paroxysmal atrial fibrillation, and a long-standing history of leg ischemia with rest pain when sleeping. He had undergone several surgical procedures over the past few years, including a femoropopliteal bypass with a Propaten graft above the knee in his right leg 2 years before the INP-treatment.

Within a few months following surgery, the patient experienced acute thrombosis of the graft and embolism distally in the leg arteries. Subsequently, a leg ulcer appeared on the patient's anterior tibia. The ulcer was treated unsuccessfully with a partial dermal skin graft from the patient's left thigh.

The patient regularly visited the wound clinic at the University Hospital to receive conventional wound care for 2 years prior to participating in the study. His maximal claudication distance was 100 m at a self-selected pace due to severe pain in his right calf. The main concern was his poor arterial leg inflow. Upon vascular examination, the patient had intact femoral artery pulses bilaterally, but no pulses in the arteries of his right foot. Duplex color scanning revealed an occluded popliteal artery. Distally, the only open artery was the fibular artery, which was open in two-thirds of the upper leg. The patient was informed that if the pain and wound worsened or failed to heal properly, amputation of the limb might be indicated.

After 8 weeks of INP-therapy 2 h per day, the leg ulcer almost healed (Fig. 1), and the SPP measured on the patient's right anterior and posterior tibial artery angiosomes on his right foot changed from 51 to 72 mmHg and 14 to 11 mmHg, respectively. The

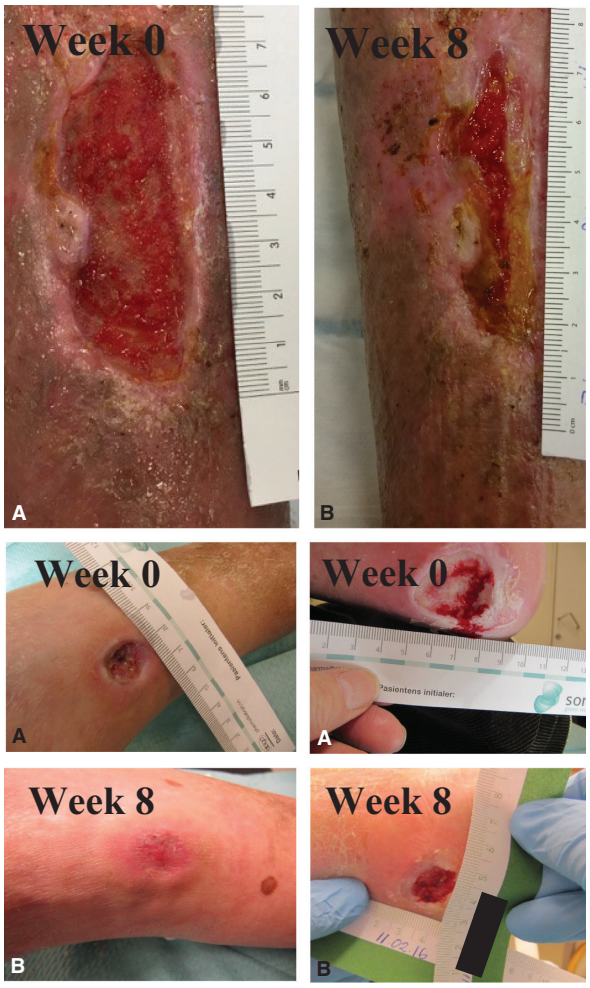

Figure 1. Upper panel: Picture of patient 1's leg ulcer before (A) and after (B) 8 weeks of INP therapy. Lower panel: Picture of patient 2's foot ulcers before (A) and after (B) 8 weeks of INP therapy. The pictures on the left are from the dorsum pedis (completely epithelialized after 8 weeks of INP therapy), and the pictures on the right are from the patient's heel (heel wound size after 8 weeks: 1.5 × 1.5 cm).

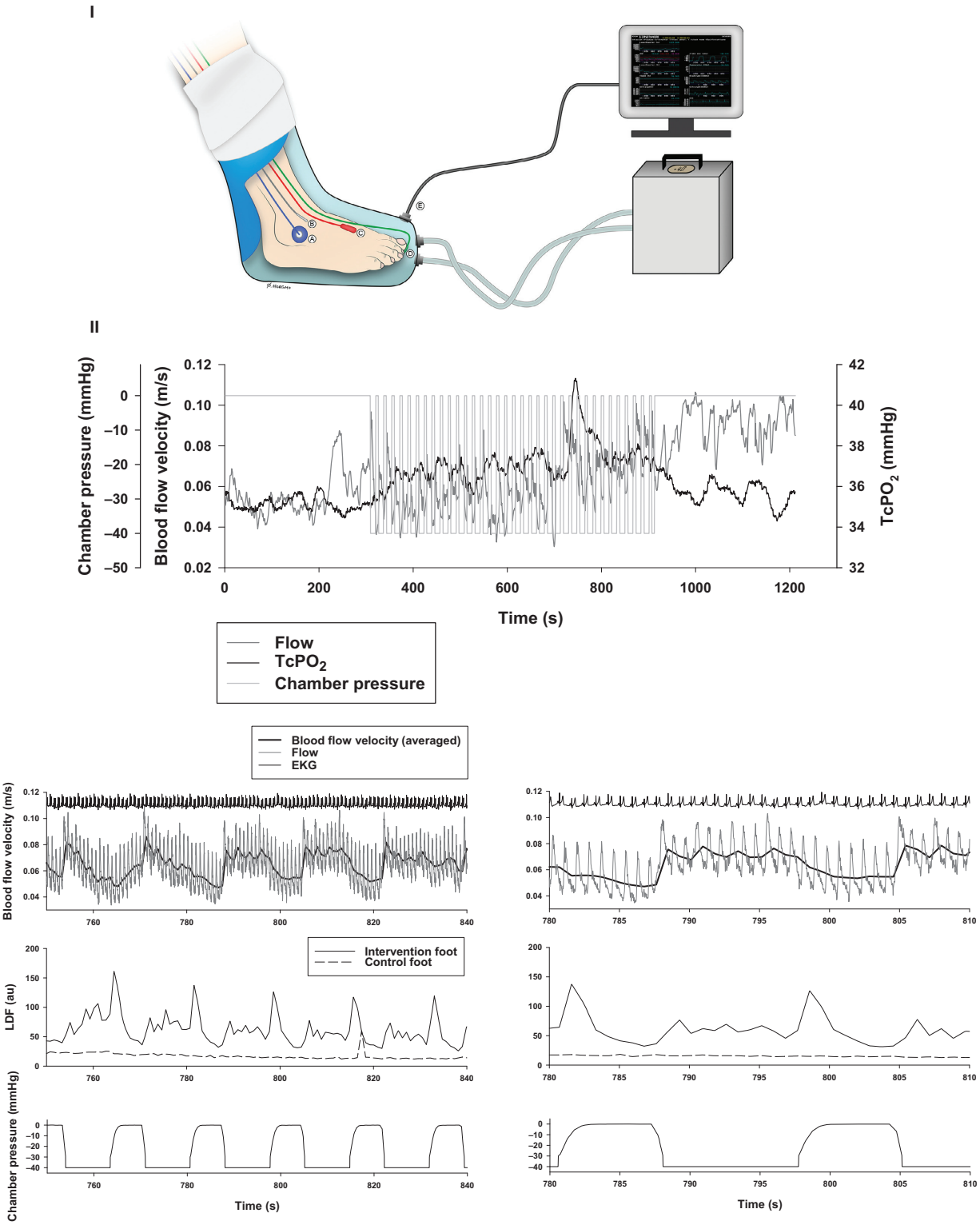

Figure 2. I: Illustration of the custom-made airtight vacuum chamber and the INP generator used by the four wound patients. The illustration shows how the probes were attached to the foot when measuring arterial blood flow velocity, laser Doppler flux (LDF), and transcutaneous oxygen pressure (TcPO2) in the foot of patient 1 during INP-therapy. (A) $TcPO_2$ probe; (B) Skin temperature probe; (C) Ultrasound Doppler probe; (D) Laser Doppler flux probe; (E) The pressure transducer from the boot interfaced with the computer. Illustration: Øystein H. Horgmo, University of Oslo. II: Measures of acute hemodynamics in patient 1 after 8 weeks of INP. The upper large panel is $TcPO_2$ and arterial blood flow in the dorsal pedis artery (flow velocity) during the whole 20-min sampling period: 5 min atmospheric pressure, 10 min INP and 5 min atmospheric pressure. Lower six panels: Beat-to-beat measures during application of intermittent negative pressure (INP) in patient 1 zoomed in from 750 to 840 sec (left) and from 780 to 810 sec (right). The panels show blood flow velocity and pulp skin flow response in the patient's right foot during application of INP. Upper panels: Blood flow velocity (ultrasound Doppler – thin lines), averaged within heartbeats (thick lines). Middle panels: Laser Doppler flux. Lower panels: Chamber pressure during INP.

patient's distal ankle PVR increased from 6 to 8 mm in the leg exposed to INP, but remained unchanged in the control leg. We also measured acute changes in transcutaneous oxygen pressure ($TcPO_2$) and blood flow velocity in the dorsal pedis artery in the lower limb exposed to INP. Laser Doppler flux was measured in the pulp of both big toes during a 10-min sequence of INP (Fig. 2).

Patient 2

The patient was a 61-year-old Caucasian male with paraplegia and complete paralysis at the level of Th6-7. He was a non-smoker and non-diabetic with a large deep wound on his heel, and a smaller wound at the dorsum of the foot (Table 1). The size and depth of the wounds had been unchanged for the past 2 years. His left leg was amputated several years ago above the knee after two similar wounds did not heal and frequently became infected. The patient received wound care and had been closely followed up by a wound nurse for the past 2 years.

After 8 weeks of INP-therapy 2 h per day, the patient's SPP in the big toe pulp increased from 44 to 95 mmHg, and his ABPI increased (Table 1). The wound on the dorsum of the foot healed completely, while the size of the wound on the heel was almost healed after 8 weeks of INP treatment (Table 1 and Fig. 1).

Discussion

In these cases involving patients with hard-to-heal leg and foot ulcers, we observed that INP therapy improved ulcer healing considerably. Foot perfusion improved after completion of 8 weeks of INP-therapy (Table 1). Blood flow and wound oxygenation are key determinants of wound healing (Sen 2009; Castilla et al. 2012; Thomas 2013), we therefore measured blood flow and $TcPO_2$ during an INP sequence in patient 1. In this patient, the increase in blood flow velocity and laser Doppler flux was followed by a rise in $TcPO_2$ (Fig. 2).

The clinical conditions of the two patients with the lowest foot perfusion at inclusion, patient 1 and 2, improved to the extent that amputation is no longer imminent. Patient 1 stated that he now experiences no rest pain and has improved his claudication distance and his quality of life. Our findings support the results of Smyth (1969), who reported improved wound healing in patients with leg ulcers after INP-therapy.

The mechanisms for the observed clinical effects and increased blood flow at onset of INP remain unknown (Fig. 2, upper panel). According to Poiseuille's law, flow (Q) between arteries and veins is proportional to the pressure gradient (ΔP) between the arteries (P_a) and veins (P_v) as follows: $Q = \Delta P\ (P_a - P_v)/R$ (Resistance), where R depends on vessel radius, vessel length and blood viscosity. If P_v suddenly changes (decreases when applying negative pressure), flow will abruptly increase. The observed initial increased flow response to negative pressure should take place only from arteries to the veins, since veins have valves which hinder backflow. This is supported by Smyth (1969), who found that the pressure in the veins and in the deep tissues of the calf followed closely the pressure within the vacuum chamber.

After a few seconds of negative pressure, we observed a decrease in flow velocity (Fig. 2), possibly due to continued inflow from the arteries and consequently, a reduction in the pressure gradient. Repetitive flow pulses may also affect biochemical factors. Cell culture models subjected to pulsatile shear stress have been shown to increase production of prostacyclin to more than twice the rate of cells exposed to steady shear stress and 16 times greater than that of cells in stationary cultures (Frangos et al. 1985). Similar findings on shear stress and cell culture models have been found with endothelial-derived relaxing factor (Cooke et al. 1990). Moreover, *flowmotion* has been suggested as an important factor in tissue oxygenation (Tsai and Intaglietta 1993). Fluctuations in blood flow induced by INP may contribute to an "artificial flowmotion" effect.

Lastly, two limitations should be addressed. First, this exploratory case report was not a placebo-controlled study with a sham intervention. The patients received traditional wound care in the hospital prior to and during

the intervention. This consisted of nutrition advice, wound debridement, and dressing to optimize healing. Second, the patients were not selected according to a randomized protocol. The patients were their own controls, as they had visited the wound clinic weekly for a minimum of 5 months prior to the study. Despite these methodological limitations, it is in our opinion unlikely that ulcers would heal spontaneously to the degree observed in the present report for all cases over 8 weeks.

In conclusion, the use of INP-therapy should be investigated further as a potential non-invasive treatment option for patients with peripheral arterial disease and hard-to-heal leg ulcers.

Ethics Approval

The experimental protocol was approved by the regional ethics committee (REK Sør-Øst 2015/1318).

Consent for Publication

All patients provided written informed consent to participate and to publish pictures of their wounds.

Availability of Data and Material

The datasets analyzed during this study is available from the corresponding author on reasonable request.

Acknowledgments

The authors thank the patients for their participation. We also thank wound nurses Heidi Jeanette Viken and Turid Østengen Aasgaard for their cooperation and encouragement. INP-generators and custom-made vacuum chambers were provided by Otivio AS. We thank Annie Bersagel for language editing, and Øystein Horgmo at the Medical Photography Section at the Institute of Clinical Medicine, University of Oslo, for help with the illustration of the test setup.

Conflict of Interests

This case study was supported in part by The Norwegian Research Council and Otivio AS. ØHS is employed by Otivio with funding from the Norwegian Research Council. Otivio AS owns and has the commercial rights to the INP technology used in the study. IM is the CSO, a co-founder and a shareholder of Otivio AS. None of the other authors have any personal conflicts of interest – financial or otherwise. The authors alone are responsible for the content and writing of the paper.

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III

PLOS ONE

RESEARCH ARTICLE

The acute effects of lower limb intermittent negative pressure on foot macro- and microcirculation in patients with peripheral arterial disease

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Abstract

Background

Intermittent negative pressure (INP) applied to the lower leg and foot increases foot perfusion in healthy volunteers. The aim of the present study was to describe the effects of INP to the lower leg and foot on foot macro- and microcirculation in patients with lower extremity peripheral arterial disease (PAD).

Methods

In this experimental study, we analyzed foot circulation during INP in 20 patients [median (range): 75 (63-84yrs)] with PAD. One leg was placed inside an air-tight vacuum chamber connected to an INP-generator. During application of INP (alternating 10s of -40mmHg/7s of atmospheric pressure), we continuously recorded blood flow velocity in a distal foot artery (ultrasound Doppler), skin blood flow on the pulp of the first toes (laser Doppler), heart rate (ECG), and systemic blood pressure (Finometer). After a 5-min baseline sequence (no pressure), a 10-min INP sequence was applied, followed by 5-min post-INP (no pressure). To compare and quantify blood flow fluctuations between sequences, we calculated cumulative up-and-down fluctuations in arterial blood flow velocity per minute.

Results

Onset of INP induced an increase in arterial flow velocity and skin blood flow. Peak blood flow velocity was reached 3s after the onset of negative pressure, and increased 46% [(95% CI 36–57), $P<0.001$] above baseline. Peak skin blood flow was reached 2s after the onset of negative pressure, and increased 89% (95% CI 48–130), $P<0.001$) above baseline. Cumulative fluctuations per minute were significantly higher during INP-sequences compared to baseline [21 (95% CI 12–30)cm/s/min to 41 (95% CI 32–51)cm/s/min, $P<0.001$]. Mean INP

authors IM & ØHS, the funder (Otivio AS) did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. ØHS is a PhD student at the University of Oslo, Faculty of Clinical Medicine, employed by Otivio. The specific roles of IM & ØHS are articulated in the 'author contributions' section.

Competing interests: The study was supported in part by Otivio AS. ØHS is a PhD student at the University of Oslo, Faculty of Clinical Medicine, employed by Otivio. IM is the CSO, co-founder and a shareholder of Otivio AS. IM and ØHS own shares and have options in Otivio AS. Otivio AS has developed and is responsible for commercialization of the FlowOx (INP) system used in the current study. IM, ØHS and JH have submitted a patent application (pending application): Post-surgical occlusion treatment recovery and rehabilitation therapy (the patent application has not yet been granted and therefore, does not have an assigned number) owned by Otivio for a use of INP technology in a related field of application to the research addressed in this paper. Otivio AS owns several issued patents, and IM is an inventor on multiple patents applications related to FlowOx [Name: Device for applying a pulsating pressure to a local region of the body and applications thereof, US 8821422 B2; Name: Portable patients temperature adjustment apparatus and method, US 20140128781 A1; Name: Vacuum-stiffening device for use in applying pulsating pressure therapy to a patient, Number: WO 2015082955 A1, Name: Pressure chamber support structure for medical pressure therapy (pending application, no number available); Name: Seal for medical pressure therapy (pending application, no number available)]. Otivio AS has received a research grant from The Research Council of Norway to fund an industrial PhD project which in part funds the PhD project including salary of ØHS (NFR grant number: 241589). Other than providing support in the form of salaries for authors IM & ØHS, the funder (Otivio AS) did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. None of the other authors have any personal conflicts of interest – financial or otherwise. This does not alter our adherence to all the PLOS ONE policies on sharing data and materials.

blood flow velocity increased significantly ~12% above mean baseline blood flow velocity [(6.7 (95% CI 5.2–8.3)cm/s to 7.5 (95% CI 5.9–9.1)cm/s, $P = 0.03$)].

Conclusion

INP increases foot macro- and microcirculatory flow pulsatility in patients with PAD. Additionally, application of INP resulted in increased mean arterial blood flow velocity.

Introduction

Lower extremity atherosclerotic peripheral arterial disease (PAD) results in reduced tissue perfusion and inadequate oxygen delivery due to narrowing of the arterial tree [1]. Insufficient blood flow may result in inadequate nutritive supply with tissue breakdown and ulceration, a process that if untreated may lead to frank gangrene and loss of the extremity [1]. The majority of treatment strategies for PAD are geared towards impeding progression of the disease and increasing blood flow [1]. The first-line treatment for PAD focuses on behavioral changes, such as reducing risk factors and improving exercise performance through ambulatory exercise [1–3]. If behavioral changes fail, the next line of therapy is revascularization through endovascular or open surgery [4]. Unfortunately, the current treatment strategies are insufficient for many PAD patients [5–7].

We have recently demonstrated that intermittent negative pressure (INP) applied to the lower leg and foot increased arterial and skin blood flow in the foot in healthy volunteers, with only minor changes in central hemodynamics [8]. Using INP with short negative pressure (-40mmHg) pulses lasting 10 to 30s, the increase in flow was due to INP-induced increases in flow pulsatility [8]. Furthermore, an eight-week pilot study on four PAD patients with hard-to-heal lower limb ulcers indicated that repetitive use of INP may improve long-term clinical outcomes, such as wound healing and foot perfusion [9]. Despite promising results for the use of INP to increase peripheral circulation in healthy volunteers, the acute effects of INP on PAD patients' peripheral circulation are unknown.

The aim of the present study was therefore to describe the effects of applying INP to the lower leg and foot on foot macro- and microcirculation and central hemodynamics in patients with PAD. Based on our previous findings in healthy volunteers [8], we hypothesized that INP would increase flow pulsatility in the foot and increase arterial and skin blood flow.

Materials and methods

Eligible PAD patients were recruited from the outpatients' clinic at Section of Vascular Investigations, Oslo University Hospital, Aker between October 2015 and April 2016. The experimental protocol was approved by the *Regional Committees for Medical and Health Research Ethics* in Norway (protocol number: 2014/1967) and performed in accordance with the Declaration of Helsinki. Written and oral informed consent was obtained from all patients before the experiment began.

Participants

The twenty-one patients recruited into the study had symptomatic lower extremity PAD (Fontaine stage grade II [1], with obstructions of the aortoiliac (n = 2) and femoropopliteal (n = 19) arteries, and an ankle-brachial pressure index (ABPI) at rest of $\leq$0.9. Exclusion criteria were: i)

chronic respiratory insufficiency or ii) eczema and psoriasis or iii) uncontrolled hypertension or iv) recent (12 months) vascular, abdominal, cardiothoracic, or lower limb orthopedic surgery.

All patients refrained from eating two hours before the experiment and from using alcohol, tobacco or caffeine on the experimental day.

Anthropometric and diagnostic measurements

We measured the patients' height and weight before a 5-min rest. Thereafter, we obtained their supine resting ABPI [10]. The limb with the lowest systolic ankle pressure was chosen as the test leg during the experiment (limb exposed to INP). The pulse volume recording curve was measured in both lower limbs with an air-plethysmography cuff placed on each subject's distal ankle (Stranden MacroLab, STR Teknikk, Aalesund, Norway). The amplitude correlates with arterial inflow to the extremity, and a sequential reduction in pulse wave amplitude from the thigh to the calf signifies the presence of a flow-limiting lesion in the more proximal arterial segment [11].

The patients' exercise tolerance was tested through an exercise treadmill test using a Skinner-Gardner protocol [12] on a treadmill (Lexco, LGT 7716S, Taeyoung Co, Korea). Briefly, the protocol uses a progressive graded workload with a constant speed of 3.2 km/h, and an increased grade of 2% every 2-min [12]. The maximal walking distance was recorded.

Experimental design

The patients were comfortably clothed and seated in an armchair for 20-min before the experiment started. After attaching the probes to the foot (Fig 1), the patients' feet were covered with loose, non-elastic wool socks to keep them under thermoneutral ambient conditions. A light blanket covered the patient's body to avoid cooling.

During the experiment, the patients were asked to sit with an approximate angle of 130˚ in their knee and hip joints (Fig 1). The test leg was placed in a rigid molded polyethylene custom-made vacuum chamber coupled to a pressure control system (FlowOx™; Otivio AS, Oslo, Norway). The vacuum chamber had internal padding to allow insertion of a leg with probes and prevent pressure points on the leg and skin. Only the posterior part of the lower leg touched the padding (Fig 1). The vacuum chamber was sealed just below the knee with a thermoplastic elastomer (TPS-SEBS) to allow for application of negative pressure. The contralateral leg was placed outside the vacuum chamber in atmospheric pressure. Pressures were continuously monitored throughout the study using a calibrated pressure probe attached to the vacuum chamber. The experimental sequences are shown in Fig 2. Briefly, 5-min baseline registrations were performed with no pressure manipulation (atmospheric pressure). Thereafter, INP was applied for 10-min using an oscillation protocol with cycles of 10s -40mmHg negative pressure and 7s atmospheric pressure [8]. Finally, a 5-min post-INP sequence at atmospheric pressure was recorded. All experiments were conducted in a quiet and temperature-controlled environment (25.1±1.5˚C) to allow patients to obtain their thermoneutral zone and to reduce sympathetic stress that could create artifacts [13]. Data were analyzed in custom-made software (REGIST 3, Morten Eriksen, University of Oslo, Oslo, Norway).

Signal acquisition and analysis

Arterial blood flow velocity was measured continuously in the dorsalis pedis/posterior tibial artery with a 10MHz pulsed Doppler probe (SD-50; GE Vingmed Ultrasound, Horten, Norway). The ultrasound beam was positioned centrally at either the dorsum pedis or posterior to the medial malleolus of the ankle, and attached with surgical adhesive tape (Micropore

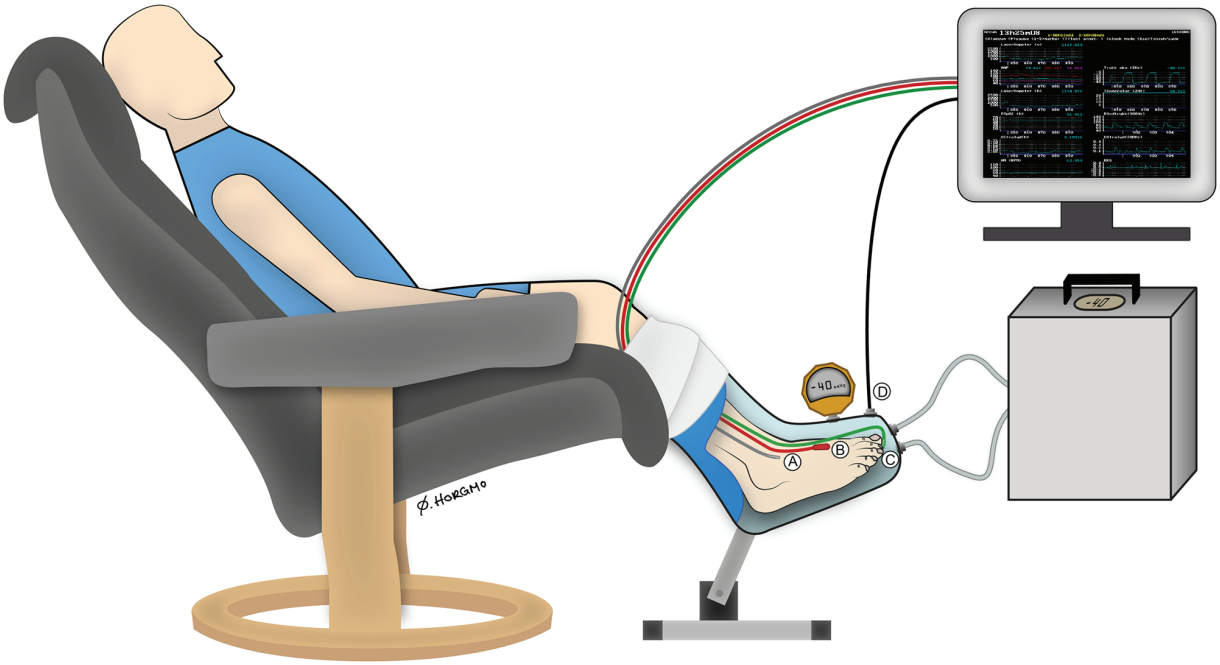

Fig 1. Illustration of the experimental setup with probes attached to the foot. The patient's test leg was placed in the custom-made vacuum chamber interfaced with the pressure control system. The vacuum chamber was sealed around the patient's leg below the knee. The contralateral leg was placed outside the vacuum chamber, acting as a control in atmospheric pressure. A: Skin temperature probe; B: Ultrasound Doppler probe; C: Laser Doppler flux probe; D: Pressure transducer from vacuum chamber interfaced with the computer. An additional external calibrated pressure gauge (Fluke, 700G Series, Everett, WA, USA) was used for calibration (REGIST 3). Illustration: Øystein H. Horgmo, University of Oslo.

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Surgical Tape, 3M, MN, US). If it was not possible to acquire a flow signal in a patient's dorsalis pedis artery, the posterior tibial artery was used.

Pulp skin blood flow was measured using laser Doppler fluxmetry (LDF; Periflux PF 4000, Perimed AB, Järfälla, Sweden), giving a semi-quantitative measurement of changes in cutaneous peripheral microcirculation expressed in arbitrary units (AU) [14]. After preparing the

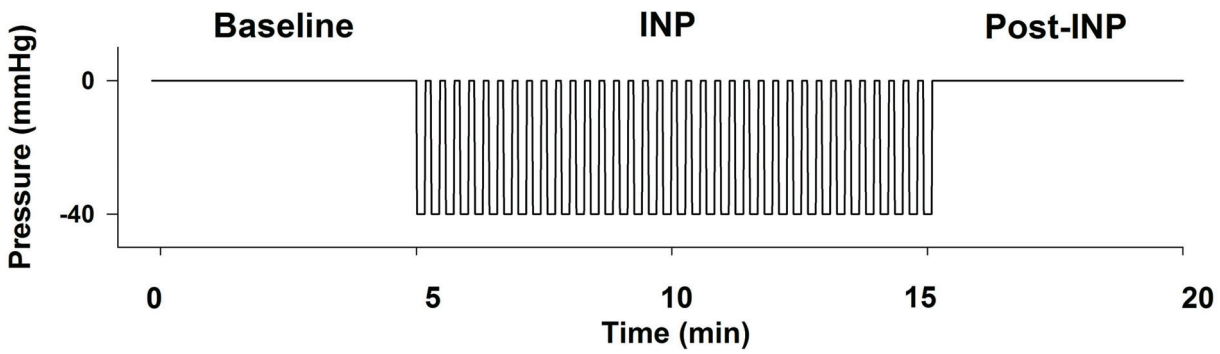

Fig 2. Description of the study sequences and the intermittent negative pressure (INP) applied. The three sequences were as follows: (i) 5-min baseline (no pressure), (ii) 10-min INP, followed by (iii) 5-min post-INP (no pressure). One pressure cycle equals 10s -40mmHg negative pressure and 7s atmospheric pressure. INP = intermittent negative pressure.

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skin with an alcohol swab, we attached LDF probes (404–1; Perimed AB, Järfälla, Sweden) bilaterally to the pulps of the first toes.

We recorded arterial pressure continuously from the third finger of the right hand using a photoplethysmographic volume-clamp method (Finometer; FMS Finapres Medical Systems BV, Amsterdam, The Netherlands). Finometer pressures were calibrated with measurements from an automated sphygmomanometer (Solar 8000i, GE-Marquette Medical Systems, Inc., Milwaukee, USA) at the beginning of each experiment. Skin temperature was continuously measured within the vacuum chamber on the foot in close proximity to the dorsalis pedis artery using an Analog Devices AD590 temperature transducer (STR Teknikk, Aalesund, Norway).

Analog signals for all measurements were sampled at 300Hz and averaged for each heartbeat gated by the R-waves of the 3-lead ECG using custom-made software (REGIST 3). The patients were instructed to avoid moving and talking throughout the sampling period. All experiments were performed by the same researcher.

Tolerability of negative pressure

At the end of each experiment, the patients rated their level of discomfort experienced during INP using a verbal numerical pain rating scale 0–10, ranging from zero (no pain or discomfort) to ten (worst imaginable pain).

Statistical analysis

Patient characteristics are described by mean (standard deviation) or median (range). A mixed effects model was applied taking into account individual subject-to-subject variation. Mean differences and 95% confidence intervals (CI) were reported from the mixed effects model regression analysis. Statistical analyses were performed using R version 3.2.4 with the "nlme" [15] and "multcomp" [16] packages (R Foundation for Statistical Computing, Vienna, Austria). For all statistical tests, a 2-tailed probability level of $P \leq 0.05$ was considered statistically significant.

Comparisons of mean values between sequences

For each subject, mean flow velocity (cm/s) and laser Doppler flux (AU) were calculated for each sequence (baseline, INP and post-INP). To remove disturbances in the transition zones, the first and last 10s of each sequence were removed before analysis. Effects of the INP and post-INP sequences compared to baseline were evaluated in a mixed effects regression model with subject as a random effect by assigning variables to each sequence.

Changes over time within one negative pressure cycle

To examine the change in flow velocity over time within one negative pressure cycle, flow velocity was binned within each second after the onset of negative pressure (second 0 through 17), giving a total of 706 INP cycles for the 20 patients. The effect of each second after start of negative pressure compared to the mean flow velocity during the baseline sequence was evaluated in a mixed effects model with subject as a random effect by assigning variables to each second.

Cumulative up-and-down fluctuations in arterial blood flow velocity

To evaluate the fluctuations in arterial blood flow velocity within the sequences, the differences between the flow velocity of each heartbeat and the previous were calculated. The sum of the

absolute values of these differences were calculated within each sequence for each subject, and averaged per minute. This gave the average cumulative variation in blood flow velocity per minute. By assigning variables to the sequences, the effects of each sequence were calculated in a mixed effects model and estimates and confidence intervals calculated as above.

Results

Twenty-one patients were included in the study. One patient was excluded to due inadequate, Doppler signals. As a result, 20 patients with PAD Fontaine stage grade II were included in the final analysis (Table 1). None of the patients used vasoactive drugs at the time of study. Blood flow velocity was measured in the dorsal pedis artery in all but two patients, where measurements were performed in the posterior tibial artery due to occluded dorsal pedis arteries.

Mean arterial blood flow velocity and mean laser Doppler flux

Compared to the baseline sequence, mean arterial blood flow velocity was higher during INP sequence and in the 5-min post-INP sequence (Table 2). There were no statistically significant effects of INP on mean laser Doppler flux in the control leg or test leg (Table 2).

Flow changes over time within one INP cycle

INP induced increase in blood flow velocity and flux pulsatility. The mean arterial blood flow velocity for each second during the INP sequence is presented in Fig 3. Estimates of changes in blood flow velocity (cm/s) and laser Doppler flux (AU) over time during one pressure cycle (17s) relative to the baseline sequence for all negative pressure cycles in all patients (706 pressure cycles analyzed in total) are presented in Fig 4. Peak arterial blood flow velocity was

Table 1. Patients' characteristics, n = 20[†].

Variables	
Age (yr)	75 (6.6)
Body mass (kg)	80 (12)
Height (cm)	174 (6.3)
BMI (kg/m^2)	26 (3.4)
ABPI test leg	0.57 (0.18)
ABPI control leg	0.61 (0.21)
PVR test leg (mm)	5.2 (2.9)
PVR control leg (mm)	6.7 (3.8)
Systolic blood pressure (mmHg)	142 (23)
Diastolic blood pressure (mmHg)	72 (9)
Verbal Numerical Rating Pain Scale (0–10)	0 (0)
Maximal walking distance (m)	341 (107–1070)
Smoking (current / history / never), n (%)	6 (30%) / 13 (65%) / 1 (5%)
Diabetes mellitus (type 2), n (%)	4 (20%)
Statins, n (%)	18 (90%)
Platelet inhibitors, n (%)	19 (95%)
Prior revascularization in test leg, n (%)	12 (60%)

BMI, Body Mass Index; ABPI, Ankle-Brachial Pressure Index; PVR, Pulse Volume Recording

Values are mean (SD)

Values are median (min-max)

[†]18 males and 2 females

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Table 2. Physiological parameters for all the PAD patients (n = 20) during baseline, INP, and post-INP. Estimates are mean and 95% CI.

Parameters	Baseline		INP			Post-INP		
	Estimate	95% CI	Estimate	95% CI	*P*-value	Estimate	95% CI	*P*-value
Blood flow velocity, (cm/s)	6.7	5.2 to 8.3	7.5	5.9 to 9.1	0.03	7.5	5.9 to 9.0	0.035
LDF test leg (AU)	422	176 to 668	446	200 to 692	0.42	421	175 to 667	0.98
LDF control leg (AU)	646	388 to 903	659	402 to 918	0.66	662	404 to 920	0.62
Skin temperature (˚C)	33.6	33.0 to 34.3	33.8	33.1 to 34.4	0.02	34.1	33.4 to 34.7	<0.001
MAP, (mmHg)	88	81 to 94	86	79 to 91	0.1	84	78 to 91	0.02
Heart rate (beats/min)	65	61 to 69	65	61 to 69	0.86	65	61 to 69	0.58

LDF, Laser Doppler flux; PAD, Peripheral Arterial Disease; INP, intermittent negative pressure; MAP, Mean arterial pressure; AU, Arbitrary Units
P-values are for comparisons to baseline

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reached after 3s, with an increase of 46% (95% CI 36% to 57%, $P<0.001$) compared to baseline (Fig 4). Peak laser Doppler flux in the test leg was reached after 2s, with an increase of 89% [(95% CI 48% to 130%, $P<0.001$)] compared to baseline. Laser Doppler flux measured in the leg not exposed to INP (control leg) did not change during the pressure cycles [peak increase: 11% compared to baseline (95% CI -1% to 25%, $P<0.08$)] (Fig 4 and Table 2).

Cumulative up-and-down changes in arterial blood flow velocity during INP

Mean cumulative fluctuations were 21 (95% CI 12 to 30)cm/s/min in the baseline sequence. This increased to 41 (95% CI 32 to 51, $P<0.001$ compared to baseline)cm/s/min during the INP sequence. The post-INP sequence was not significantly different from baseline, 24 (95% CI 15 to 33, $P = 0.38$ compared to baseline)cm/s/min. Cumulative fluctuations during the sequences are presented in Fig 5, and blood flow velocity and flux responses in one patient during the whole 20 minute experiment are presented in Fig 6.

Central hemodynamics—Heart rate and blood pressure

Heart rate and blood pressure are presented in Table 2. There were no clinically significant differences in mean arterial pressure (MAP) or heart rate (HR) during between the INP or post-INP sequences compared to baseline.

Discussion

The main finding in the present study was that applying INP (-40mmHg) to the lower leg and foot in PAD patients increased fluctuations in arterial blood flow velocity compared to baseline (Figs 3 and 4). INP induced an increase in peak arterial blood flow velocity of 46% at the onset of negative pressure compared to average baseline blood flow velocity (S1 Video). A corresponding increase in pulp skin blood flow fluctuations in the test foot was observed during INP (Fig 4), while skin blood flow on the foot outside the vacuum chamber did not change (Fig 4). The cumulative fluctuations in arterial blood flow velocity during the INP sequences were significantly higher compared to the baseline sequences (Fig 5). This fluctuation response reflects increased blood flow pulsatility during INP compared to baseline (Figs 3 and 4).

The proposed theoretical foundation for applying negative pressure intermittently instead of constantly is that constant negative pressure applied to a body part causes venous distension and reduces blood flow locally via the venoarteriolar reflex [17, 18]. Accordingly, we recently demonstrated that constant negative pressure of -40mmHg applied to the lower leg and foot

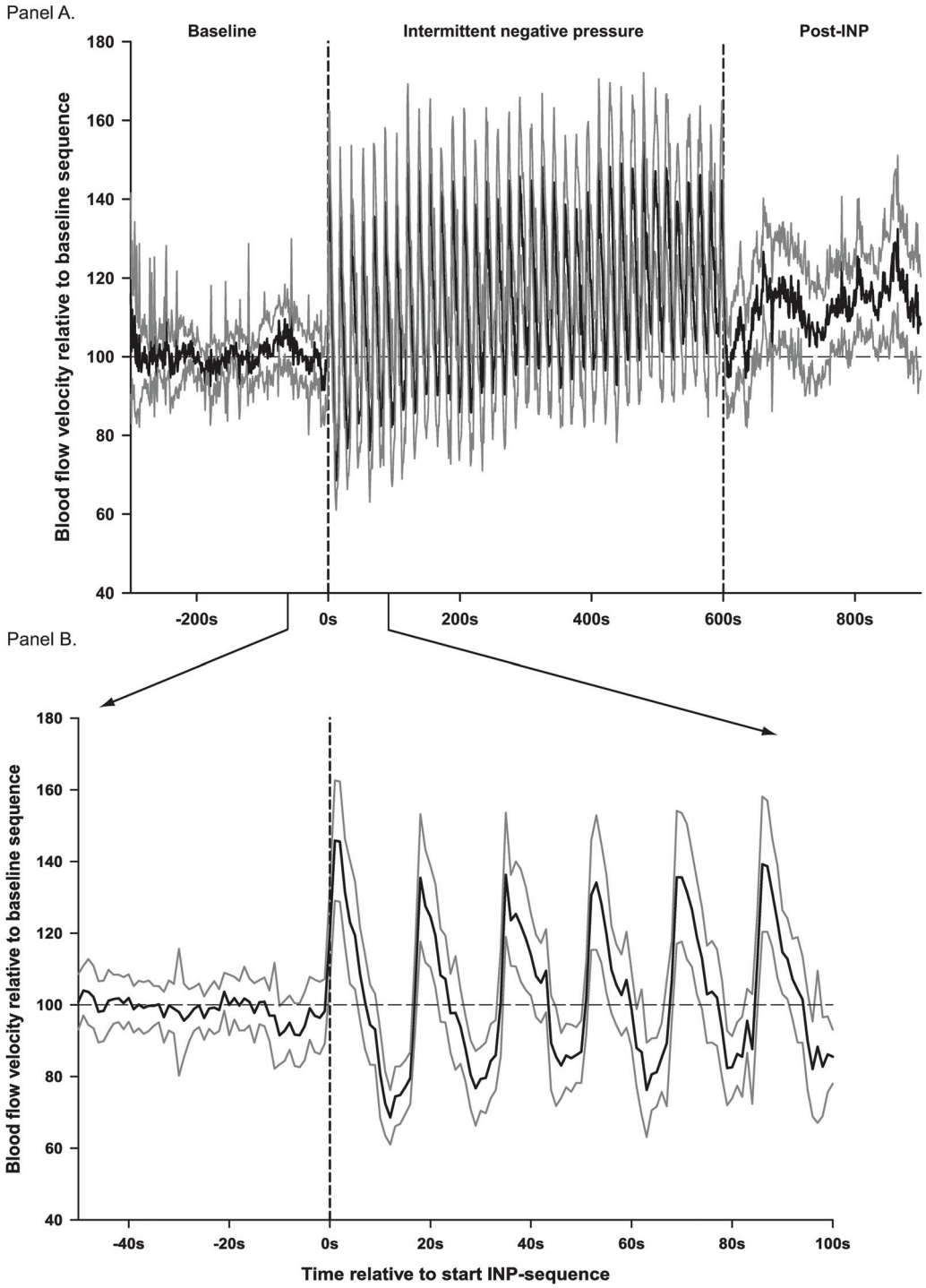

Fig 3. Arterial blood flow velocities (cm/s) for each second relative to each subject's mean baseline value. Black lines are mean values with 95% confidence intervals for each second as grey lines. Panel A shows the whole 20-min experiment. Panel B shows a section at the end of baseline and beginning of INP.

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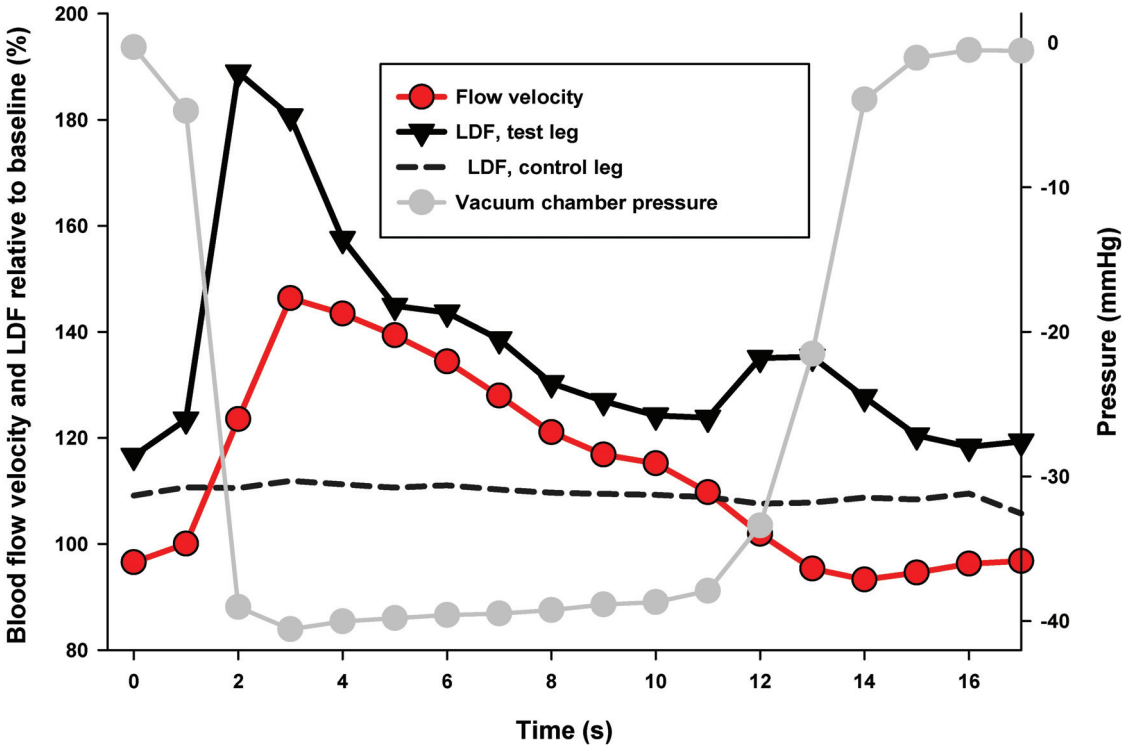

Fig 4. The effects of time for the first 17s (one pressure cycle) after onset of negative pressure. Effect estimates are for all the INP-cycles aggregated within and between subjects relative to each subject's mean baseline value. Blood flow velocity (cm/s), Laser Doppler Flux, LDF (AU) measured in test leg and control leg (shown as dashed line); Vacuum chamber pressure (mmHg, right y-axis).

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resulted in a decreased arterial blood flow velocity, skin blood flow, and skin temperature in the foot of healthy volunteers [8]. The same negative pressure applied intermittently resulted in increased blood flow pulsatility and an increase in mean blood flow velocity [8]. In these healthy volunteers, we found that INP increased peak blood flow velocity in foot arteries by 44% during the first few seconds after onset of negative pressure [8]. The present study on PAD patients observed that blood flow pulsatility increased as seen in the study on healthy volunteers [8]. This shows that the peripheral vasculature in the PAD patients may have a comparable ability to increase macro- and microcirculatory foot circulation during INP as healthy subjects.

In order to regulate vascular tone and tissue perfusion, endothelial cells sense blood flow and react to this mechanical stimulus, releasing vasoconstriction and vasodilation substances [19]. Considering the effects of flow shear stress on the release of biochemical mediators such as prostacyclin [20] and endothelial-derived substances [21–23], INP-induced flow pulsatility may cause beneficial vascular adaptions after repetitive use. Compared to nonpulsatile flow conditions, endothelium-derived nitric oxide release is significantly enhanced during conditions of flow pulsatility in the peripheral vasculature in vivo [24], and in vitro using cell cultured endothelial cells [25]. The relatively modest (~12%) increase in mean arterial blood flow velocity during INP sequences in the PAD patients (Table 2) is similar to that observed in healthy volunteers (~8%) [8]. This finding is somewhat surprising given the marked increase in blood flow pulsatility (up-and-down fluctuations) during INP, as shown in Figs 3 and 4.

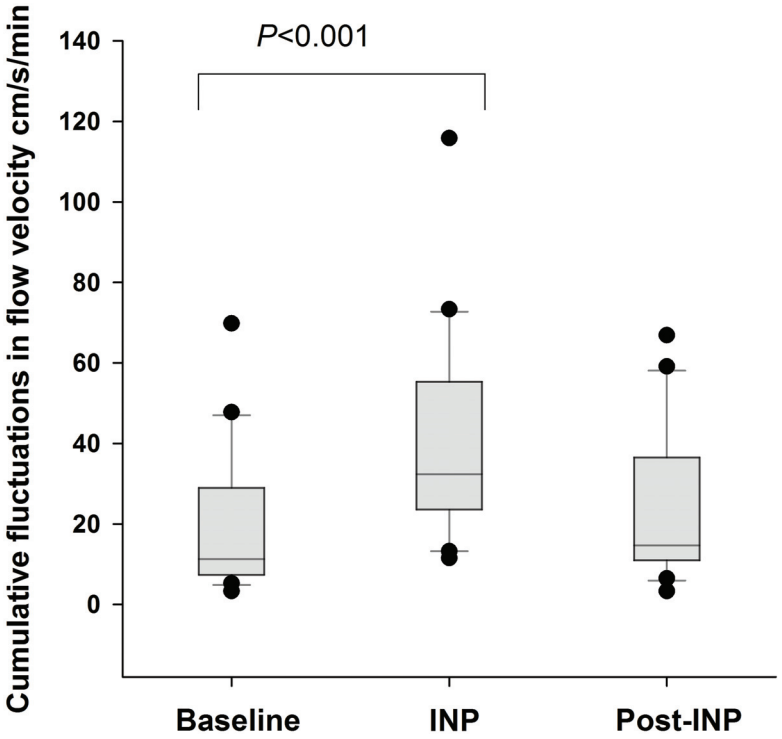

Fig 5. Fluctuations (cumulative up-and-down) in blood flow velocity during baseline (no pressure), INP, and post-INP (no pressure) sequences. The cumulative up-and-down are calculated per minute. Whiskers are 10th and 90th percentiles.

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These blood flow velocity fluctuations caused the blood flow velocity, after reaching a maximum at about 3-4s after onset of negative pressure, to gradually decline below baseline values after about 12s (Fig 4). This decline in blood flow velocity below baseline values will reduce the mean blood flow velocity during the INP sequence. This gradual reduction in blood flow velocity relative to baseline described in Figs 3 and 4 may be due to venous distension, which elicits the venoarteriolar reflex [17, 18]. Alternatively, the decline in blood flow velocity may be due to a decreased pressure gradient, which is in turn caused by continued arterial inflow during the negative pressure cycles [9].

In the present study, there was also a significant (~12%) increase in mean blood flow velocity during the 5-min post-INP sequence compared to baseline (Table 2 and Fig 3, panel A), suggesting that INP had a prolonged effect on blood flow. This increase in post-INP blood flow velocity was observed despite no significant increase in cumulative up-and-down fluctuations in blood flow velocities compared to baseline (Fig 5). This increase in mean arterial blood flow velocity during the post-INP sequence may be due to INP-induced shear stress. Flow-mediated vasodilation may therefore explain some of the positive clinical outcomes after INP treatment reported in earlier studies [26–29], and our own observations [9]. The potential impact of long-term INP use on flow-mediated vasodilation should be the subject of further studies.

Reports on "suction devices" to the lower limb for the treatment of tissue ischemia date back to the early 19th century [26, 30–35]. These initial studies were limited by the lack of objective outcome variables (i.e. use of self-reported walking distance), small sample sizes, and weak study designs [36, 37]. In the late 1960s, Smyth [27] performed the first analyses of the

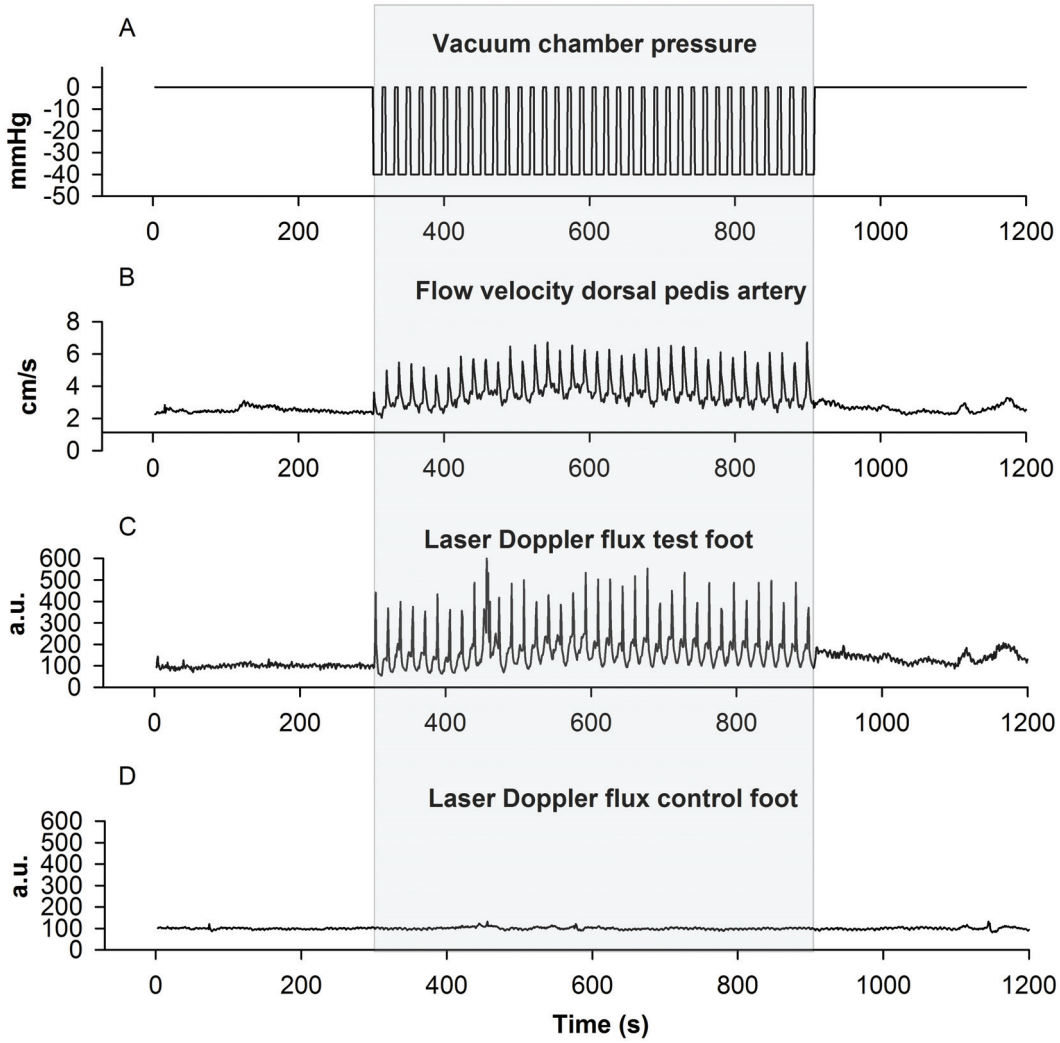

Fig 6. An example of blood flow velocity and laser Doppler flux responses in one of the patients (age: 64 years; Fontaine stage IIB; ABPI test leg: 0.21, ABPI control leg: 0.29, PVR test leg: 1mm, PVR control leg: 4mm) during the 20 minute experiment: 5 min baseline (atmospheric pressure), 10 min INP, and 5 min post-INP (atmospheric pressure) sequences. Panel A: Pressure within the vacuum chamber; Panel B: Blood flow velocity (ultrasound Doppler); Panel C: Laser Doppler flux in the test foot; Panel D: Laser Doppler flux in the control foot not exposed to INP. All panels' timelines are from 0 to 1200s (x-axis).

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isolated effects of INP (-150mmHg) on femoral artery flow. This study reported data on acute blood flow measurements in femoral arteries and veins, as well as calf inflow in patients with peripheral vascular disease and intermittent claudication. Smyth [27] used plethysmography to measure calf inflow in 11 patients following 6 weeks of INP treatment two times per week, reporting a 282% increase in mean resting blood flow in the calves, and a 300% increase in peak reactive hyperemia after 2 minutes of occlusion. This study measured arterial blood flow velocity changes during INP, but did not provide any data on INP's effect on foot circulation, skin blood flow or central hemodynamics [27]. The protocol Smyth [27] described was later replicated by Gill et al. [38] in a four week intervention study on three patients with Raynaud's disease of the hands and eight patients with intermittent claudication. Gill et al. [38] reported

increased systolic ankle pressure and calf muscle blood flow (133^{XE} clearance technique) in claudicants, as well as increased hand skin blood flow in the patients with Raynaud's disease.

In a randomized crossover trial, Himmelstrup et al. [28] reported beneficial effects of intermittent negative and positive pressure applied to the lower body, with pressure oscillations lasting 5-10s (Vacusac, Intl. aps, Denmark). In this study, 22 patients with intermittent claudication were randomized to either 2 months Vacusac (active treatment) or placebo. The main findings were that patients receiving active treatment significantly increased pain-free and maximal walking distance on the treadmill and increased their ABPIs [28]. Importantly, the increased ABPIs were followed by a decrease in systemic blood pressure [28]. When patients were crossed over, those receiving active treatment again improved, while the placebo group did not. Similar improvements in ABPIs, pain-free and maximal walking distance on 34 patients with intermittent claudication have also been described by Mehlsen et al. [29] in a double-blinded randomized trial comparing 25 Vacusac treatments to 25 placebo treatments over two months. Mehlsen et al. [29] also reported significant increases in the ADP threshold for platelet aggression after treatment.

In a recent case study on four PAD patients (ABPI $\leq$0.60) with long-lasting (6–24 months) hard-to-heal leg ulcers, we observed—in addition to improved wound healing—similar results to those of the aforementioned Himmelstrup and Mehlsen studies [28, 29] on foot perfusion. We observed increases in ABPIs after eight weeks of INP therapy two hours per day [9]. Our findings on the acute effect of INP on increased macro- and microcirculatory circulation in the present study on PAD patients may explain the clinically beneficial effect of ambient pressure therapy applied to the lower limb on wound healing and ischemic limbs, consistent with the aforementioned studies.

In contrast to the commonly used negative pressure wound therapy (NPWT), which applies high negative pressure (-50 to -125mmHg) to the local wound environment to remove excessive fluid [39], the INP method used in the present study applies mild negative pressure to a large area of the lower limb (Fig 2). This could theoretically facilitate increased arterial inflow in all six angiosomes of the lower leg and foot due to mechanical dilation of the tissue and vessels exposed to INP. In contrast to the findings that INP resulted in increased flow pulsatility in arteries and the small vessels distally in the foot in patients with PAD, NPWT has shown to decrease blood flow in close proximity to the negative pressure area [40]. Additionally, whether NPWT increases tissue perfusion remains controversial [41, 42].

Limitations of the study

There are some limitations to the present study. Firstly, blood flow velocities were used to describe changes in arterial flow in the foot in PAD patients. We were not able to measure the diameter of the dorsalis pedis and tibial posterior arteries within the vacuum chamber during INP. However, pulsatile diameter in small arteries has been found to be very stable [43]. We therefore believe it is reasonable to assume that the changes observed in blood flow velocity during INP reflect changes in blood flow in the foot artery. If the vessel diameter increased during negative pressure, then our measurements would underestimate arterial blood flow velocity during INP.

Secondly, skin temperature was measured on the dorsum of the foot, and the temperature sensor was isolated from the surrounding air with several layers of adhesive tape (Micropore Surgical Tape, 3M, MN, US). It is possible that the tape did not adequately isolate the probe and that an increase in skin temperature could be due to increased temperature inside the "air pocket" within the vacuum chamber. Leakage results in different pressure curves, which could give a different physiological response. Nevertheless, we observed increased skin temperature

together with increased peak blood flow velocity and laser Doppler flux during the pressure cycles. Skin blood flow measured with laser Doppler flux has been found to be highly correlated with local skin temperature in subjects exposed to temperatures between 23 to 36˚C (r = 0.87) [44]. Together, these findings indicate increased tissue perfusion of the foot during INP.

Thirdly, our study had an unequal number of males and females. Differences between sexes in the INP-induced flow responses may be a subject for further studies.

Conclusions

In the present study on patients with PAD, we observed increased peripheral blood flow pulsatility in the foot during application of INP to the lower limb. The mean arterial blood flow velocity increased slightly during INP and the increase sustained for at least a 5-min period after termination of INP. The increased laser Doppler flux and skin temperature observed during the negative pressure cycles reflect increased blood flow to the small vessels of the skin. The observed increase in macro- and microvascular flow during INP in patients with ischemic limbs may improve tissue perfusion, wound healing and patency after revascularization. Future studies are warranted to explore the working mechanisms of INP and possible clinically relevant effects after repetitive exposure to INP.

Supporting information

S1 Video. A live Doppler signal response during intermittent negative pressure applied to the lower leg and foot in a patient with peripheral arterial disease (Fontaine stage II). The video shows a typical live Doppler signal response when measuring blood flow velocity in the most distal foot artery (*arteria dorsalis pedis*). Twenty seconds into the recording, the negative pressure is applied for 10 second followed by a 7 second period of atmospheric pressure. The sequence is repeated with the negative pressure (-40 mmHg) starting again after 37 seconds and 54 seconds.
(MP4)

S1 Data. The dataset includes all of the 20 PAD patients.
(TXT)

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