Effects of treatment with lower extremity intermittent negative pressure for peripheral artery disease

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ABBREVIATIONS

PAD	Peripheral artery disease
INP	Intermittent negative pressure
IC	Intermittent claudication
CLI	Critical limb ischemia
PWD	Pain free walking distance
MWD	Maximal walking distance
PVR	Pulse-volume recording
LDL	Low density lipoprotein
HDL	High density lipoprotein
ABI	Ankle-brachial index
CT	Computed tomography
MRA	Magnetic resonance angiography
SET	Supervised exercise therapy
RCT	Randomized controlled trial
CONSORT	Consolidated standards of reporting trials
NYHA class	New York Heart Association functional classification
ELISA	Enzyme-linked immunosorbent assay
VCAM-1	Vascular adhesion molecule-1
ICAM-1	Intracellular adhesion molecule-1
ADMA	Asymmetric dimethylarginine
SDMA	Symmetric dimethylarginine
vWF	von Willebrand Factor
HPLC	High-performance liquid chromatography
NO	Nitrogen monoxide
CI	Confidence interval
SD	Standard deviation
SEM	Standard error of the mean
MAP	Mean arterial pressure
LDF	Laser Doppler flux

ANCOVA	Analysis of covariance
ANOVA	Analysis of variance

LIST OF PAPERS

I Hoel H, Høiseth LØ, Sandbæk G, Sundhagen JO, Mathiesen I, Hisdal J. The acute effects of different levels of intermittent negative pressure on peripheral circulation in patients with peripheral artery disease. Physiological Reports. 2019;7(20):e14241.

II Hoel H, Pettersen EM, Høiseth LØ, Mathiesen I, Seternes A, Hisdal J. A randomized controlled trial of treatment with intermittent negative pressure for intermittent claudication. J Vasc Surg. 2021;73(5):1750-8.e1.

III Hoel H, Pettersen EM, Høiseth LØ, Mathiesen I, Seternes A, Seljeflot I, Hisdal J. Effects of intermittent negative pressure treatment on circulating vascular biomarkers in patients with intermittent claudication. Vasc Med. 2021:1358863x211007933. Epub ahead of print.

IV Hoel H, Pettersen EM, Høiseth LØ, Mathiesen I, Seternes A, Hisdal J. Lower extremity intermittent negative pressure for intermittent claudication. Follow-up after 24 weeks of treatment. Ann Vasc Surg. 2021:S0890-5096(21)00272-7. Epub ahead of print.

THESIS SUMMARY

In peripheral artery disease (PAD), arterial blood flow to the extremities is impeded. Globally, more than 235 million people are affected by the disease, and the prevalence is increasing. Clinically, the severity of PAD ranges from asymptomatic disease, to intermittent claudication or atypical extremity pain during exercise, to critical limb ischemia characterized by rest pain, tissue loss and gangrene. Exposure of the extremity to cyclic pressure changes increases the macro- and micro circulation and has been described since the early 20th century. However, it has more recently become a treatment option, as new treatment devices for the application of intermittent negative pressure (INP) to the lower leg have been developed. The overall aim of this thesis was to investigate the clinical and physiological effects of lower extremity INP treatment in patients with PAD.

In an experimental study (paper I), we investigated the acute effects of different levels of INP on the macro- and microcirculation in the leg in 16 patients with PAD. In a randomized double-blind, sham-controlled trial (paper II and III), we investigated the clinical, physiological, and quality of life related effects of treatment with lower extremity INP for one hour, twice daily for 12 weeks, in 72 patients with intermittent claudication. In a follow-up trial (paper IV), we investigated the clinical effects of treatment with INP for one hour, twice daily for 24 weeks in 10 patients with intermittent claudication.

Application of -40 and -60 mmHg INP significantly increased arterial and skin blood flow compared to atmospheric pressure. INP of -10 mmHg did not significantly increase blood flow, and was therefore chosen as the INP level for the sham device in the randomized controlled trial. Treatment with -40 mmHg INP for one hour, twice daily for 12 weeks increased pain free walking distance compared with sham treatment with -10 mmHg INP (estimated treatment effect 50 m; 95% CI [11, 89]; p=0.014). For the patients with the most symptomatic disease (baseline pain free walking distance < 200 m) there were significant differences between the groups in both pain free- and maximal walking distance after 12 weeks, favouring the treatment group over the sham control group (estimated treatment effect 42 m; 95% CI [2, 83]; p=0.042, and estimated treatment effect 62 m; 95% CI [5, 118]; p=0.032, respectively). Of the patients randomized to the treatment group, 25/31 (81%) had a reduction in circulating levels of von Willebrand Factor (vWF) after 12 weeks, compared to 17/30 (57%) in the sham control group (p=0.043). Within the treatment group there was a significant reduction in the concentration of vWF of (mean [SEM]) -

11% [4] (p=0.019) after 12 weeks. No significant effects were observed on resting- and post-exercise ABI, resting- and post-ischemic calf blood flow, quality of life parameters or levels of circulating vascular biomarkers between the groups after 12 weeks of treatment. After treatment with -40 mmHg INP for 24 weeks, there was a significant increase in both pain free- and maximal walking distance, compared to baseline (p=0.006 and p=0.012, respectively). The main increase in pain free walking distance occurred during the first 12 weeks of treatment, whereas the main increase in maximal walking distance occurred from 12 to 24 weeks of treatment.

In conclusion, application of -40 mmHg INP acutely increased arterial and skin blood flow in the leg. Treatment with -40 mmHg INP for one hour, twice daily for 12 weeks increased walking distance compared to sham treatment. A significant proportion of the patients in the treatment group had a reduction in vWF, and the levels of vWF were reduced in the treatment group after 12 weeks, which might indicate a beneficial effect on arterial endothelial activation and endothelial injury. The positive effects on walking distance seemed to persist from 12 to 24 weeks of INP treatment.

1. BACKGROUND

1.1 Atherosclerosis

One of the first descriptions of atherosclerosis dates back to the time of Leonardo DaVinci (1452-1519), who after performing autopsy of an old man, noted that the death was caused by "failure of the blood and of artery that feeds the heart" [1]. The modern concept of the process of atherosclerosis was introduced by Rudolf Virchow in 1858, who described the inflammatory nature of the atherosclerotic plaque, and recognized atherosclerosis as an active process of tissue reaction [2]. In 1908, Ehrlich and Mechnikov received the Nobel prize for their work on adaptive and innate immune responses [3], however, the understanding of these concepts in the context of atherosclerosis was not adopted until almost a century later. In most of the 20th century, atherosclerosis was considered to be a cholesterol storage disease characterized by collection of cholesterol and cellular debris in the arterial wall [4]. In the 1970s there was an increasing focus on the proliferation of smooth muscle cells in the formation of atherosclerotic plaques [5, 6], and the observation that the atherosclerotic process could be initiated by mechanical, immunologic or chemical injury to the arterial endothelium formed the response-to-injury hypothesis [7-10]. However, atherosclerosis as an inflammatory disease has only been adopted for the last 30 years [11, 12].

Principally, the atherosclerotic lesions occur in large and medium sized arteries. A healthy middle to large artery in the body consists of three histologically different layers: the tunica intima, the tunica media, and the tunica adventitia (Fig 1). The tunica intima consists of one continuous layer of endothelial cells supported by an elastic collagenous layer of variable thickness. The tunica media consists of a layer of variable ratios of smooth muscle cells and elastic components. The tunica adventitia is a collagenous layer that may contain vasa vasorum which provides blood supply to the vessel wall in larger arteries [13]. The endothelial cells regulate exchanges between the blood stream and the surrounding tissues, and are able to respond to physical and chemical stimuli by the release of factors that affect vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation and vessel wall inflammation [14]. Under ordinary circumstances, the endothelium resists the adhesion of inflammatory components in the blood. However, when the endothelium becomes activated by an atherogenic or proinflammatory stimulus, an upregulation of the expression of adhesion molecules promotes recruitment of monocytes and T lymphocytes that adhere to the arterial wall [15].

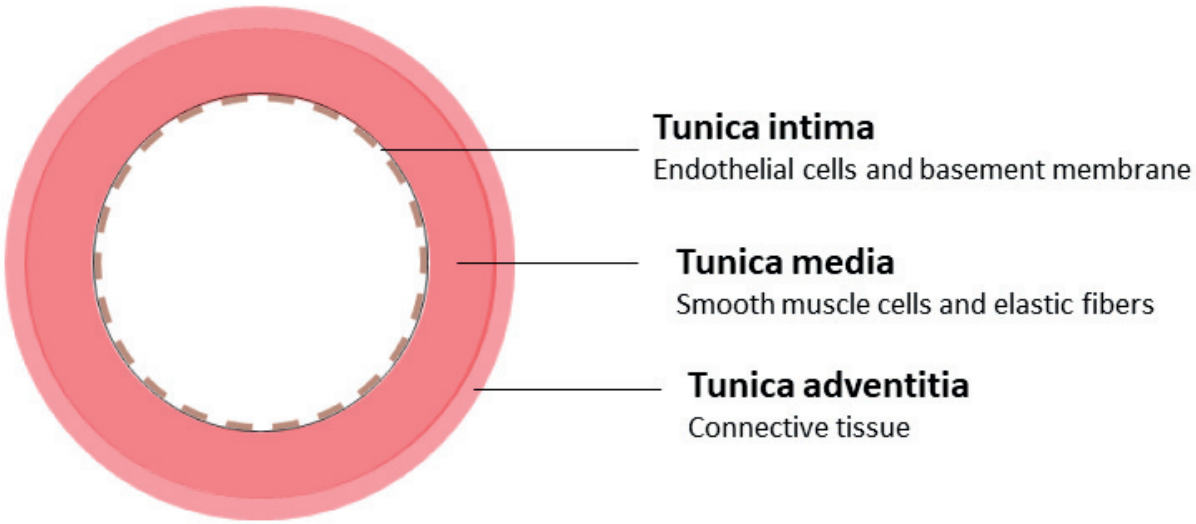

Fig 1. Cross section of a healthy middle to large artery. The figure is drawn by Henrik Hoel.

In the early phase of atherosclerosis, the endothelial cells release chemoattractant cytokines that stimulates monocytes and T lymphocytes to enter the intima by a process known as diapedesis through the junctions between the endothelial cells [16]. Inside the intima, the monocytes are transformed into macrophages expressing receptors for internalizing lipoproteins. Lipid loaded macrophages, known as foam cells, replicate inside the intima, and secrete proinflammatory cytokines and reactive oxygen species that amplify the local inflammatory response causing progression of the atheromatous lesion [17].

Histologically, the first recognizable sign of atherosclerosis is the fatty streak, which is an aggregation of lipid rich macrophages and T lymphocytes within the intima. The fatty streak may occur from an early age and have been described in autopsy materials from infants and young children [18]. As the atherosclerotic process progress, the fatty streaks develop into atherosclerotic plaques, causing narrowing of the arterial wall. When the narrowing of the arterial wall affects the blood flow through the area to the extent that downstream tissues suffer from ischemia, it is denoted as a significant stenosis.

Activated phagocytes produce matrix metalloproteinases that degrade the extracellular matrix that support the atherosclerotic plaque, hence, are central in the thrombotic complications of atherosclerosis. Destabilization of the fibrous plaque, may lead to rupture or fissures particularly in the plaque margins, resulting in haemorrhage into the plaque, thrombosis and occlusion of the artery when the blood get in contact with pro-coagulant proteins in the plaque

produced by the macrophages [19]. Cell death of macrophages at this location forms the necrotic core often observed in the atherosclerotic lesion.

1.2 Peripheral artery disease

1.2.1 Definitions and classifications

The definition of peripheral artery disease varies in the literature from comprising all entities which result in obstruction of blood flow in the arteries, exclusive of the coronary and intracranial vessels [20], to all states that causes stenosis or occlusion of the lower- or upper-extremity arteries [21]. Throughout this thesis the latter definition is used. Atherosclerosis is by far the most common cause of PAD, however several non-atherosclerotic occlusive disorders may also cause PAD, including fibromuscular dysplasia, different forms of vasculitis, arterial entrapment, radiation injury, and trauma. PAD may be asymptomatic, or present with symptoms of intermittent claudication (IC) or atypical limb pain, critical limb ischemia (CLI) or acute limb ischemia [21].

The word “claudication” is derived from Latin “claudicare” which means to limp. The term alludes to the Roman emperor Claudius (reigned AD 41 to 54) who suffered from physical impairment caused by sickness at an early age, which made him walk for a short distance, then stop and stand before start walking again [22]. IC is a common symptom in patients with PAD characterized by muscle discomfort in the lower limb provoked by exercise that is relieved by rest, and was first described by the French veterinarian surgeon Bouley in 1831, in a limping horse suffering from occlusions of the femoral arteries of the posterior limbs [23]. The French neurologist Charcot described the syndrome in humans in 1858 and introduced the term IC [24].

The first classification system for PAD was published by Fontaine et. al in 1954 [25]. The Fontaine classification system grades the disease in to four stages, and is solely based on the clinical presentation, without additional diagnostic tests (Table 1). Another commonly used classification system for PAD is the Rutherford classification [26]. For chronic PAD, the Rutherford classification stratifies symptomatic disease into six categories based on clinical presentation, and objective measurements such as treadmill tests, ankle pressure, toe pressure and pulse-volume recording (PVR). Both classification systems are commonly used for research

purposes. They may be used to direct patient management, however, are not routinely used in the clinical setting.

Table 1. Classification of peripheral artery diseases according to Fontaine's stages [25] and Rutherford's categories [26], adapted from the Inter-Society Consensus for the Management of Peripheral Artery Disease (TASC II) [27].

Fontaine		**Rutherford**		
Stage	Clinical	Grade	Category	Clinical
I	Asymptomatic	0	0	Asymptomatic
IIa	Mild claudication	I	1	Mild claudication
IIb	Moderate to severe claudication	I	2	Moderate claudication
		I	3	Severe claudication
III	Ischemic rest pain	II	4	Ischemic rest pain
IV	Ulceration or gangrene	III	5	Minor tissue loss
		III	6	Major tissue loss

1.2.2 Epidemiology

Over the last decades, there has been an epidemiological transition from morbidity and mortality caused by communicable diseases to chronic non-communicable diseases. Cardiovascular diseases are significant contributors to morbidity and mortality in both high- and lower-income countries today [28, 29]. PAD affects more than 235 million people worldwide [30], and is the third leading cause of cardiovascular morbidity after coronary heart disease and cerebrovascular disease [31]. PAD is uncommon among younger people, but the prevalence increases with increasing age, and affects a substantial proportion of the elderly population. The estimated

prevalence ranges between 3-10% in the total population, increasing to 15-20% in persons over 70 years [27].

The natural course of PAD differs between patients. About 20-50% are asymptomatic, 10-35% have typical IC symptoms, 30-40% have more atypical leg pain, and 1-3% presents with CLI as their first sign of the disease [27]. CLI is a state of arterial insufficiency leading to chronic inadequate tissue perfusion at rest and represents the end stage of PAD. Clinically, the condition is characterized by rest pain, tissue loss and/or gangrene. CLI may develop secondarily to IC, or primarily without any prior PAD symptoms. For both asymptomatic and symptomatic patients with PAD that have not developed CLI, the expected 5-year outcome is the same, with a mortality of 10-15%, in which 75% dies from cardiovascular causes. The risk of deterioration to CLI are independent of symptoms; 70-80% experience a stable situation with IC, 10-20% develop worsening of claudication symptoms, and 5-10% progress to CLI within five years. When CLI is present, the yearly mortality rate is 25%, 45% are alive with two limbs, and 30% go through an amputation within one year [27].

Generally, persons with PAD have a 2- to 4-fold higher mortality risk compared to those without PAD [32, 33]. This is mainly explained by the increased risk of cardiovascular events, and reflects the underlying systemic process of atherosclerosis. Hence, many patients with PAD are also suffering from other cardiovascular diseases such as coronary heart disease, cerebrovascular disease, and chronic kidney disease. It is estimated that about 60% of the patients with PAD, also have concomitant coronary disease or cerebrovascular disease [34].

IC is a common clinical symptom of PAD that may cause significant functional disability. The prevalence of IC increases from around 3% in patients at age 40 to 6% in patients aged 60. In younger age groups, claudication is more common in men, but at older age groups there is less difference between men and women [27]. Data from the Framingham study showed an overall biennial incidence of IC of 7.1 per 1000 for men and 3.6 per 1000 for women, with a sharp increase in incidence with age up to 75 years [35].

1.2.3 Risk factors

As atherosclerosis is the main aetiology for PAD, the major risk factors are similar to other cardiovascular diseases, and includes smoking, diabetes, hypertension and dyslipidaemia. However, the impact of each risk factor seems to differ per arterial site [36].

Smoking affects endothelial function, oxidative processes, platelet function, fibrinolysis, inflammation, lipid oxidation and vasomotor function [37], and is a major risk factor for cardiovascular diseases in general, and particularly for PAD. There is a strong dose-response relationship between pack-years of smoking and risk of PAD, and the association between PAD and smoking persists after smoking cessation. However, the risk of PAD is nearly 3-fold higher among current smokers than ex-smokers, emphasizing that smoking cessation is never too late [38].

Diabetes mellitus is strongly associated with an elevated risk of PAD, and the association is even stronger with more severe and longstanding diabetes [39]. The increased risk and accelerated development of atherosclerosis in patients with diabetes are caused by metabolic abnormalities resulting in hyperglycaemia, dyslipidaemia, oxidative stress and increased inflammation [40]. The outcomes for PAD patients with diabetes are worse, with five times increased risk of amputation, and three times increased risk of mortality compared to PAD patients without diabetes [41].

Hypertension entails increased stress on the arterial wall and is associated with increased risk of atherosclerosis and development of PAD. The systolic blood pressure seems to be more important for the development of PAD than the diastolic blood pressure [34]. In two large population-based studies, hypertension was second only to current smoking as the most contributable risk factor for developing PAD [38, 42].

Hypercholesterolemia is a significant risk factor for atherosclerosis, and development of PAD. The cholesterol of the atherosclerotic plaques is derived from low-density lipoprotein (LDL) cholesterol. LDL particles are circulating in the blood, and high levels of circulating LDL is associated with more rapidly development of atherosclerosis [43]. In a large prospective study, hypercholesterolemia demonstrated a high, graded, and independent association with PAD [38]. High-density lipoprotein (HDL) cholesterol is protective against the development of PAD, and an elevated ratio of total cholesterol to HDL cholesterol is strongly associated with the disease [44].

1.2.4 Diagnostics

The ankle-brachial index (ABI) is a simple, non-invasive, and inexpensive test for PAD. In addition to the medical history and a general physical examination, ABI should be the initial diagnostic test for PAD, and may be the only test required to establish the diagnosis and to initiate treatment [45]. ABI is calculated by dividing the higher systolic pressure measured with

ultrasound Doppler in the dorsalis pedis artery and the tibialis posterior artery, by the higher of the left and right arm systolic pressure measured in the brachial artery [46]. The threshold for diagnosing PAD is an ABI ≤ 0.9. This threshold was originally based on studies from the 1960s that estimated a sensitivity and specificity to detect PAD > 90% compared to conventional angiography [47, 48]. However, a more recent systematic review confirmed a specificity of 83-99%, but a lower sensitivity of 15-79%, especial lower in elderly and diabetic patients [49]. ABI is a strong marker of generalized atherosclerosis and cardiovascular risk, and ABI ≤ 0.9 is associated with a 2-3-fold increased risk of cardiovascular death [36]. An ABI ≥1.4 represents stiff leg arteries due to calcification of the tunica media. This is most commonly seen in elderly and diabetic patients and is also associated with an increased risk of cardiovascular mortality [36].

For symptomatic patients with a normal or borderline resting ABI, the ABI after leg exercise may indicate if a hemodynamically significant stenosis is present. With exercise, the systemic systolic pressure increases. The blood is distributed to the exercising muscles, and vasoconstriction occurs in non-exercising limbs and organs. In healthy subjects this leads to a mild decrease in ABI, that is normalized within a short time after exercise. In patients with even moderate PAD, the ankle pressure drops more during leg exercise compared with healthy subjects, and the recovery time is prolonged [46]. A decrease in ABI ≥20% after walking exercise is considered diagnostic for PAD [27].

Although less frequently used in clinical practice, PVR may increase the diagnostic accuracy of PAD when combined with ABI. PVR is measured with an air-plethysmography cuff wrapped around the extremity at certain levels. In healthy individuals, arterial inflow to the extremities is pulsatile, leading to measurable changes in limb volume with each cardiac cycle. In patients with PAD, the waveform of the PVR becomes dampened, with a delayed or diminished peak [50].

Anatomical assessment of the localization and distribution of the atherosclerotic lesions are generally indicated in highly symptomatic PAD patients when revascularization is considered, or for follow up after revascularization. Duplex ultrasound is a dynamic examination, that is used to detect and localize vascular lesions, and to quantify the extent and severity by measuring changes in blood velocity. Computed Tomography (CT) angiography and Magnetic Resonance Angiography (MRA) gives anatomic overviews of the arterial tree. CT angiography has the advantages of short examination time and relatively wide availability. The

drawback of CT angiography is the lack of hemodynamic data, exposure to radiation, and the use of iodine-based nephrotoxic contrast agents. MRA has a higher soft tissue resolution, which may be an advantage in peripheral artery imaging, especially more distally in the arterial tree. Motion artefacts are more frequent, the gadolinium-based contrast is nephrotoxic, and metal components that is not MRA compatible may be contraindicated. Direct catheter angiography relies on a contrast agent injected into the artery of interest through a catheter, that combined with x-ray examinations can pinpoint areas of stenosis or occlusion, and is mainly used in combination with endovascular treatment.

1.2.5 Circulating vascular biomarkers

According to the Biomarker Definitions Working Group, a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" [51]. Biomarkers are widely used in clinical practice and in research for diagnostic, predictive or prognostic purposes, and for monitoring diseases and responses to treatments. They may also give new insights into pathophysiological mechanisms. Atherosclerotic activity is associated with altered levels of circulating biochemical substances indicative of vascular inflammation, endothelial injury, endothelial function, and atheromatous plaque instability, that may serve as biomarkers for PAD [17, 52-56].

1.3 Treatments for peripheral artery disease

1.3.1 Best medical treatment

For many years, the treatment of PAD was mainly focusing on symptomatic relief and to improve functional status [57]. However, in the 1950s, increasing attention was brought to the fact that patients with symptomatic PAD had a significant increased risk of mortality [58]. From the 1990s, prospective studies have shown that only a minority of the patients experience a progression of the disease from IC to limb loss, but that the presence of PAD, symptomatic or asymptomatic, is a major predictor for coronary heart disease and cerebrovascular disease [32, 59-61]. Today, the treatment of patients with PAD includes two aspects: management of cardiovascular risk factors, and management of leg symptoms. All patients diagnosed with PAD should be

offered best medical treatment, which includes cardiovascular risk modifying measures in terms of smoking cessation, pharmacological secondary prevention, and life-style modifications [36].

Patients diagnosed with PAD are recommended to use lipid-lowering agents if not otherwise contraindicated. Statin therapy reduces all-cause mortality and cardiovascular events. LDL cholesterol should be reduced to < 1.8 mmol/L or by 50% if the initial LDL cholesterol level is between 1.8 and 3.5 mmol/L [36]. However, in addition to the cholesterol lowering effect of statins, they have plaque stabilizing effects [62], and favourable effects on vasomotor function, angiogenesis, and systemic inflammation [63-65], and are recommended for patients with PAD independent of the cholesterol levels. Statin therapy may also increase walking distance in patients with IC [36].

Antiplatelet agents are recommended in patients with PAD to prevent limb related- and general cardiovascular events. Single antiplatelet therapy with aspirin or clopidogrel are recommended as the first line treatment [36]. The effects of dual antiplatelet therapy are counterbalanced by an increased risk of bleeding, but may be recommended for a time-limited period after endovascular therapy for PAD [36]. Anticoagulant agents have traditionally not been used as first line treatment of PAD, unless patients have other indications. However, the COMPASS trial recently concluded that low-dose rivaroxaban plus aspirin reduce major cardiovascular and limb related events in patients with PAD, compared with aspirin alone. Major bleeding was increased, but fatal or critical bleeding was not [66]. Hence, future guidelines may include the use of low dose anticoagulants in addition to aspirin as secondary prevention for PAD.

1.3.2 Supervised exercise therapy

Participation in supervised exercise therapy (SET) programs increase walking capacity, functional status, and quality of life [67-73], and are recommended for patients with IC. Most SET programs include at least three hours per week walking to maximal or submaximal distance with an instructor, and are more effective than home-based or unstructured exercise therapy [36]. Despite the well documented effects of SET for patients with PAD, the availability seems to be rather low. A European survey from 2012 concluded that SET programs was an underutilized tool in Europe, with only 30% of the vascular surgeons that participated answered that they had SET available as a treatment option for patients with PAD [74]. Further, SET programs face

challenges related to patient compliance and adherence. A systematic review from 2016 concluded that only 1 in 3 patients with IC were suitable for, and willing to undertake SET [75].

1.3.3 Endovascular and open surgical revascularization

For many years, open surgical revascularization in terms of thromboendarterectomy and bypass surgery, was the treatment option for symptomatic PAD, when conservative treatment failed. However, from the 1980's, less invasive endovascular treatments with percutaneous transluminal angioplasty with or without stents have been widely used. The optimal revascularization strategy is dependent on the available resources, patient comorbidity, relevant anatomy and the patients' and surgeons'/intervention radiologist's preferences. Randomized controlled trials comparing open surgical and endovascular treatment for lower extremity PAD have been criticized for patient selection, and the inability to generalize findings from highly specialized centres. However, given the availability of both endovascular and open surgical revascularization, population-based studies have shown that endovascular treatment is associated with improved long-term amputation free survival, but has a modest increased risk of subsequent interventions [76]. Revascularization with endovascular or open surgery are effective in improving leg function and reduce pain in patients with IC [77]. However, current guidelines recommend reserving this treatment for patients with disabling symptoms with substantially altered quality of life who do not respond to conservative treatment [36]. For patients with CLI, endovascular or open surgical revascularization is the corner stone treatment to prevent limb loss, reduce pain, and improve function. It is estimated that 50-90% of the patients with CLI undergo a revascularization procedure, however, for patients with severe co-morbidities or very limited chance of successful revascularization, primary amputation may be the most appropriate treatment [27].

1.3.4 Treatment with intermittent negative pressure in a historical perspective

Cupping, application of suction to areas of the skin with the use of cups, dates back to the ancient Egypt around 1500 BC [78], and is the earliest descriptions of the use of negative pressure for the treatment of disease. In ancient Greek, Hippocrates used cupping to extract injurious matter from wounds [79]. Treatments by exposure of the body or parts of the body to negative pressure was introduced in traditional medicine in the 19th century. In 1832, the physician Sir James Murray working in Dublin, Ireland described the effects of diminished atmospheric pressure on man, and

proposed a new method for the treatment of cholera by reducing the air pressure surrounding the body [80]. In 1835, the British physician William Ried Clanny published the paper "Apparatus for removing the pressure of the atmosphere from the body or limbs" in The Lancet [81], which was followed by another Lancet paper by Murray discussing potential disease states that could be treated with manipulation of the air pressure [82]. The English scientist Edgar Bluck, was the first who described the use of fluctuating negative and positive pressure to modify the arterial circulation in the extremities in 1887 [83]. At the beginning of the 1900s, the German surgeon August Karl Gustav Bier observed the natural occurring hyperaemia in diseased tissues and proposed that artificial induction of hyperaemia could promote tissue healing. He used vacuum chambers surrounding the area of the diseased tissue to induce hyperaemia to facilitate wound healing [84].

In 1917, Sinkowitz and Gottlieb reported reduced pain and improved peripheral circulation in four patients with PAD after treatment with a modified Biers hyperaemia suction apparatus [85]. In 1933, Landis and Gibbon investigated the effects of alternating suction of -120 mmHg and pressure of 80-100 mmHg on blood flow in the lower extremities in normal subjects and in patients with PAD, concluding that to obtain maximal effects on blood flow, it is advisable to have relatively brief periods of suction, and intermittent, brief periods of pressure [86]. In 1934, Herrmann and Ried published a series of 75 patients with atherosclerosis obliterans, where the affected leg was treated with rhythmic alternations of 60-80 mmHg negative pressure and 20-40 mmHg positive pressure in a boot made of pyrex glass (Fig 2). They introduced the term passive vascular exercise, and suggested that the treatment promoted the development of an adequate collateral arterial circulation [87]. Landis and Hitzrot reported positive effects of application of different levels of intermittent negative and positive pressure on wound healing, rest pain and claudication in patients with different stages of PAD in 1935 [88].

In 1969, Smyth published a paper in The Lancet, describing the effects of intermittent suction with -150 mmHg (15 seconds on, 15 seconds off) on resting and post-ischemic calf blood flow, walking distance and wound healing in 46 patients with ischemic limbs (Fig 2) [89]. Smyth used Ultrasound Doppler to measure the direct effect on the femoral artery and vein blood flow velocity before, during and after the release of suction. The femoral artery flow velocity was increased during suction, and at the end of suction Smyth described a prolonged increase in the venous outflow from the limb. Resting and post-ischemic calf blood flow were measured with

venous occlusion plethysmography in 11 patients, demonstrating a 282% increase in resting- and 300% increase in post-ischemic blood flow after 6 weeks of "suction treatment" for half an hour twice a week. Smyth also reported an improved subjectively estimated walking distance in 31 of 40 patients with IC, and improved wound healing in 3 patients with ischemic ulcers caused by Raynaud's disease [89].

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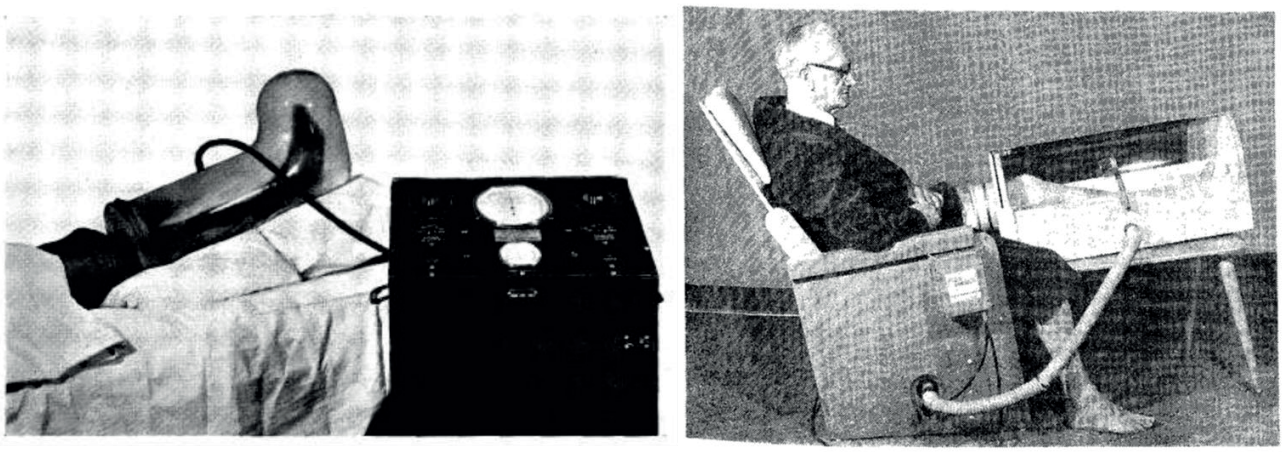

Fig 2. Device for application of intermittent positive and negative pressure by Herrmann and Reid 1934 [87] (A), and for application of intermittent negative pressure by Smyth 1969 [89] (B). Photos reproduced with permission from the copyright holders.

So far, the studies of the clinical and physiological effects of INP treatment were based on case reports and patient series. However, in the 1990s two controlled studies by Himmelstrup and Mehlsen concluded that INP treatment increased walking distance in patients with IC [90, 91]. With a cross-over design, they investigated the effects of treatment with a device called Vacusac in 22 patients with stable IC. Oscillating pressure with a mean reduction of 30% from atmospheric pressure was applied by drawing air out of a plastic bag covering the patient from the feet to up to the axilla. After 25 active treatments over a period of 2 months, the patients receiving active treatment attained a significant increase in pain free walking distance (PWD) (median [range]) from 54 m [24-107] to 99 m [30-420] and in maximal walking distance (MWD) from 99 m [36-182] to 185 m [68-1000] measured on treadmill. The patients receiving placebo treatment did not show any significant improvement in walking distance, however, when crossed

over to the active treatment they attained a significant improvement in PWD and MWD [90]. In an extension of that study, Mehlsen and Himmelstrup reported a significant increase in the threshold for adenosine diphosphate induced platelet aggregation, and an increment in fibrinolytic activity, indicating that the treatment had a positive effect on platelet aggregation in patients with PAD [91].

The principle of INP has also been used in devices for thermo regulation. INP was used to increase skin blood flow in the arm during exposure of the arm to hot or cold water. This method was effective in treating hypothermia during laparotomy for gastrointestinal surgery, and to reduce core temperature in healthy subjects [92, 93]. More recently, studies have demonstrated the acute increase in arterial and skin blood flow during and after application of INP in healthy subjects, patients with PAD, and patients with spinal cord injury [94-96], and improved wound healing in patients with PAD and spinal cord injury [97, 98].

Recently, two randomized controlled trials investigated the effects of "vacuum treatment" with a device called Vacumed for IC [99, 100]. When treated with Vacumed, the patient's lower body was positioned in an airtight vacuum chamber which was sealed around the trunk at the level of umbilicus. In the study by Hagemann et al. patients were randomized to active treatment with -37.5 mmHg oscillating (9 seconds on, 9 seconds off) negative pressure, or control treatment with -3.75 mmHg oscillating negative pressure for 30 minutes two times a week for six weeks, in addition to participation in SET [99]. In the study by Afzelius et al. patients were randomized to treatment with Vacumed for 40 minutes three times a week for six weeks in combination with home-based physical activity and lifestyle changes, or home-based physical activity and lifestyle changes alone [100]. Both studies concluded that "vacuum treatment" did not have any additional effect on walking capacity in patients with IC [99, 100].

The previous studies of the clinical and physiological effects of INP treatment are heterogenous. There are large variations in how INP was applied, levels of INP, time sequences of negative pressure and atmospheric pressure, and duration and frequency of the treatment. Common for all the previous studies, was that the treatment devices required in-hospital treatment, which restricted the opportunity for frequent treatment sessions over time.

1.3.5 The FlowOx device

FlowOx 2.0 is a CE marked medical device designed to apply INP to the extremities to improve arterial and skin blood flow (Fig 3). The foot and lower leg are treated in a pressure chamber

made of hard Polyethylene Plastics. In the bottom of the pressure chamber, the foot arch rests on a positioner, that is movable to adjust the lower legs angle inside the pressure chamber. A padding between the pressure chamber and the lower leg becomes inflated when INP is started. The pressure chamber is sealed to the leg below the knee using a thermoplastic elastomer seal. A control unit containing a pump generates INP of -40 mmHg by removing air from and venting the pressure chamber in cycles of 10 seconds negative pressure and 7 seconds atmospheric pressure. User time and failure to generate INP (e.g. air leakage) are recorded by the control unit. The device is made of lightweight components and can be operated by most patients at home.

Fig 3. The FlowOx 2.0 device. Intermittent negative pressure is generated in a pressure chamber sealed around the lower leg by a control unit containing a pump that removes air from and vents the pressure chamber. Source: Henrik Hoel, with permission from the research subject.

2. THESIS AIMS

The overall aim of this thesis was to investigate the clinical and physiological effects of lower extremity INP treatment in patients with PAD. To accommodate this, one experimental study, one clinical randomized controlled trial (RCT), and one clinical follow-up trial were conducted.

- In paper I, the main objective was twofold. First, to identify the optimal level of INP applied to the lower limb, to increase arterial and skin blood flow in patients with PAD. Secondly, to identify a lower level of INP that does not affect blood flow, appropriate for use in a sham device.
- In paper II, the main objective was to investigate the clinical effects of lower extremity INP treatment for one hour two times daily for 12 weeks in patients with IC.
- In paper III, the main objective was to investigate the potential effects of lower extremity INP treatment for one hour twice daily for 12 weeks on circulating vascular biomarkers of vascular inflammation, endothelial injury, and endothelial function.
- In paper IV, the main objective was to investigate the clinical effects of INP treatment after 24 weeks. This was a follow-up trial of patients included in the RCT.

3. MATERIALS AND METHODS

3.1 Study design

- In paper I, an experimental study design with a standardized laboratory setup was used to investigate the effects of different levels of INP on arterial and skin blood flow in patients with PAD.
- In paper II and III, a RCT design was used to evaluate the clinical and physiological effects of INP treatment in patients with IC. The study was conducted and reported in accordance with the Consolidated Standards of Reporting Trials (CONSORT) statement [101].
- In paper IV, a prospective study design was used to follow up the patients from the RCT who volunteered to continue active treatment with -40 mmHg INP for additional 12 weeks (24 weeks in total).

3.2 Study populations

In the experimental study (paper I), we recruited study subjects from the outpatient clinic at the Department of Vascular Surgery at Oslo University Hospital. Subjects with resting ABI ≤ 0.9 and symptomatic claudication, or radiologically detected PAD were included. Subjects undergoing recent (less than three months) endovascular or open surgical revascularization were considered not eligible for the study.

The RCT (paper II and III) was as multicentre trial enrolling patients from the outpatient clinics at the vascular surgery departments at Oslo University Hospital, Sørlandet Hospital, and St. Olavs Hospital, between January and September 2019. Data collection was completed in December 2019. All patients were offered best medical treatment according to the guidelines from the European Society of Cardiology and the European Society for Vascular Surgery [36]. Inclusion and exclusion criteria are listed in Table 2.

In the clinical follow-up trial (paper IV), patients who received active treatment during the 12-week intervention period in the RCT were invited to continue treatment for additional 12 weeks (24 weeks in total).

Table 2. Inclusion and exclusion criteria for the randomized controlled trial.

Inclusion criteria	Exclusion criteria
• Ankle-brachial index ≤ 0.9 or incompressible arteries in the lower leg and radiologically diagnosed peripheral artery disease • Intermittent claudication	• Scheduled for or recently (< 3 months) performed revascularization of the lower limb • Inability to operate the treatment device • Baseline MWD > 1000 m * • Severe heart disease (NYHA class IV) • Severe chronic obstructive pulmonary disease (resting dyspnoea) • Inability to make an informed consent

MWD maximal walking distance; NYHA class New York Heart Association Functional Classification. * Treadmill test, Gardner-Skinner protocol [102].

3.3 Demographic information

Demographic information was obtained from all study participants based on customized registration forms, supplemented with information from the patients' electronic medical record.

3.4 Interventions

In the experimental study (paper I), INP levels of 0, -10, -20, -40 and -60 mmHg were applied to the lower leg during sampling of hemodynamic data. The INP levels were applied in a randomized order to prevent carry over effects between the different pressure levels.

In the RCT (paper II and III), the active treatment group received an INP level of -40 mmHg, and the sham control group received an INP level of -10 mmHg for one hour in the morning and one hour in the evening for 12 weeks. The choice of -10 mmHg as the INP level for the sham device was based on results from paper I, indicating that -10 mmHg INP did not significantly affect arterial and skin blood flow compared to atmospheric pressure alone. Except from different INP levels, the two groups were treated with identical treatment devices. The patients´ most symptomatic leg decided after a baseline treadmill test was chosen as the leg to be treated throughout the intervention period. The patients that were randomized to treatment with -40 mmHg INP, and who volunteered to continue the treatment for additional 12 weeks (24 weeks in total) were included in the follow-up trial (paper IV).

3.5 Randomization and blinding

In the RCT, patients were randomized to active treatment with -40 mmHg INP or sham treatment with -10 mmHg INP in a 1:1 ratio. Labelling of the treatment devices was performed by the producer (Otivio AS), by a person not involved in the recruitment of patients, or collection of data. A statistical software was used to assign the treatment device number to the participant number when informed consent was obtained. Patients and personnel with patient contact during the study period were blinded to the group allocation, as were the medical technicians handling the blood samples and performing the laboratory analyses.

3.6 Outcome measures

3.6.1 Sampling of hemodynamic data

In the experimental study, hemodynamic data were sampled at 300 Hz, and averaged beat-by-beat, gated by the R-waves of a three lead ECG, using a custom-made software for sampling of physiological data (REGIST 3, developed by Morten Eriksen at the University of Oslo) (Fig 4). By adding the angle of insonation, and the vessel diameter, the software calculated beat-by-beat blood flow in the artery. The beat-by-beat data were converted to 2 Hz by interpolation before further analyses were conducted.

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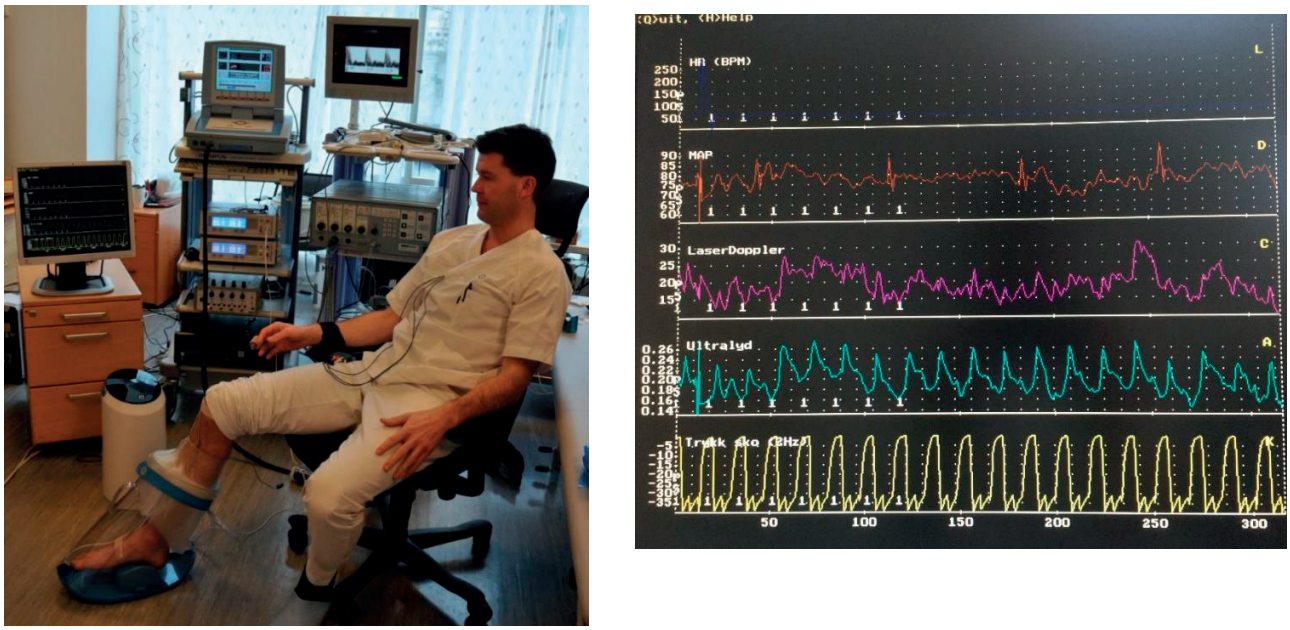

Fig 4. Sampling of hemodynamic data during application of intermittent negative pressure to the lower leg. Arterial blood velocity (dorsalis pedis artery), laser Doppler flux (pulp of the first toe) and systemic mean arterial pressure (Finometer third finger) were sampled at 300 Hz, and averaged beat-by-beat gated by the R-waves of a three lead ECG (A). Screenshot from REGIST 3 during sampling of hemodynamic data (B). Source: Jonny Hisdal/Henrik Hoel

3.6.2 Arterial blood velocity and arterial blood flow

In the experimental study, arterial blood velocity was measured during application of INP by ultrasound Doppler (SD-50, GE Vingmed Ultrasound, Horten, Norway) with the Doppler probe

attached to the dorsalis pedis artery or tibialis posterior artery, at the place where the best Doppler signal was achieved. To calculate the arterial blood flow during INP, we measured the diameter of the dorsalis pedis artery and the tibialis posterior artery using a triplex ultrasound scanner (GE LOGIQ 9 Ultrasound, Wauwatosa, Wisconsin, USA). It was not possible to measure the vessel diameters in the pressure chamber during INP. Therefore, the diameters of the dorsalis pedis and tibialis posterior arteries were measured before application of INP, and arterial blood velocity was measured during application of INP. Hence, the flow calculations were based upon the vessel diameter before application of INP.

3.6.3 Laser Doppler Flowmetry

In the experimental study, skin blood flow was measured during application of INP by a laser Doppler flow meter (PeriFlux 5000, Perimed AB, Jarfalla, Sweden) using a laser Doppler probe attached to the pulp of the first toe. Illumination of the tissue with coherent laser light which interacts with the moving blood cells leads to Doppler shifts, and by processing the frequency distribution of the backscattered light, microvascular blood perfusion can be estimated [103].

3.6.4 Central hemodynamic

In the experimental study, systemic arterial blood pressure was measured non-invasively during the experiments by a Finometer (FMS, Finapres medical systems BV, Arnhem, Netherlands) attached to the third finger of the right hand. The Finometer is a non-invasive photoplethysmography instrument that measures beat-to-beat systemic blood pressure by continuously monitoring finger arterial pressure [104]. By continuously monitoring systemic mean arterial pressure (MAP), we were able to record if the application of INP affected the central hemodynamic, and to detect if changes in MAP contributed to the fluctuations in arterial and skin blood flow during INP.

3.6.5 Walking distance

In the RCT and the follow-up trial, pain free- and maximal walking time were measured on treadmill with a ramp protocol (Gardner-Skinner protocol), using a constant speed of 3.2 km/h, starting at 0% slope, increasing by 2% every 2 minutes [102], and PWD and MWD were calculated.

The patients were asked to specify the most limiting leg after the baseline treadmill test, which was chosen as the leg to be treated throughout the intervention period.

3.6.6 Resting and post ischemic calf blood flow

In the RCT, resting blood flow was measured with strain-gauge plethysmography (Domed Filtrass Angio, Krailling, Germany) after five minutes in supine position. The strain-gauge was placed around the lower leg at the point of maximal circumference. A cuff was placed around the thigh to prevent venous return when inflated. The plethysmograph measures change in leg circumference during venous occlusion and creates a graph with complexes representing the rate of change in volume expansion for the area under the strain-gauge (Fig 5). Blood flow values are determined by calculating the slope between the complexes [105]. Post-ischemic blood flow was measured with the same strain-gauge plethysmograph after three minutes occlusion of arterial blood flow to the leg by a cuff wrapped around the thigh inflated to 250 mmHg. For safety reasons, patients who had previously undergone bypass-surgery in the leg were excluded from this examination together with patients who got unbearable pain during the three minutes occlusion period.

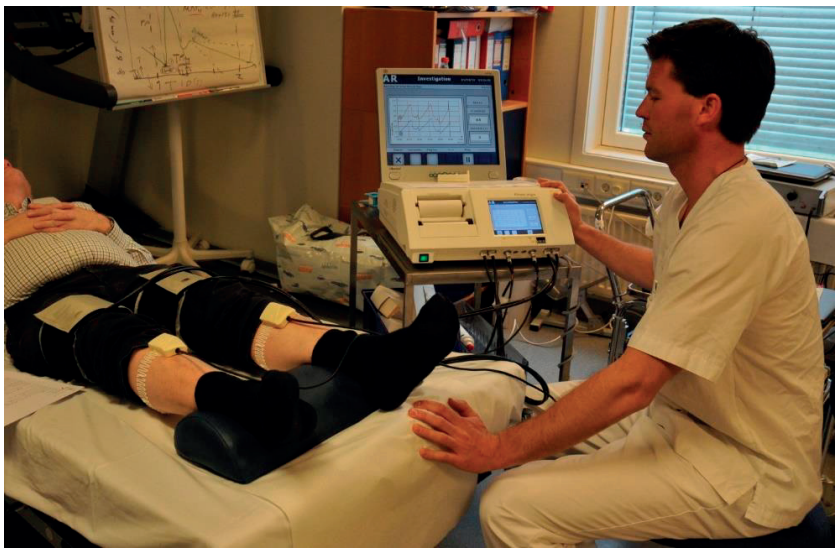

Fig 5. Arterial calf blood flow measured by strain-gauge plethysmography at rest and after three minutes of arterial occlusion. Blood flow values were calculated based on the change in volume expansion for the area under the strain-gauge placed around the calf during venous occlusion on the thigh. Source: Jonny Hisdal/Henrik Hoel, with permission from the research subject.

3.6.7 Ankle-brachial index

Resting ABI was measured after five minutes of rest, with the patient in supine position in accordance with the guidelines from the American Heart Association [46]. In the RCT and the follow-up trial, post exercise ABI was measured within one minute after the end of the treadmill test.

3.6.8 Quality of life

In the RCT, patients were asked to answer the EQ-5d-5l and Vascuqol-6 quality of life questionnaires at baseline and after 12 weeks of treatment. EQ-5d-5l is a commonly used instrument to describe and value health across a wide range of diseases [106], and consists of a descriptive system and a visual analogue scale (EQ VAS). The descriptive system comprises five dimensions: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression. Each dimension has five levels, and the patients were asked to tick off the most appropriate level for each dimension. From each patient's score in the descriptive system, an index value was calculated based on a value set validated for Denmark [107]. The EQ VAS records the patients self-rated health on a vertical scale from 0 to 100, higher score indicating better health. The Vascuqol-6 is a disease specific health-related quality of life questionnaire for patients with PAD validated for Norway [108], consisting of six disease specific items scored from one to four with a sum score ranging from six to 24, higher score indicating better health.

3.6.9 Circulating vascular biomarkers

In the RCT, venous blood samples were collected from all patients under fasting conditions between 08.00 am and noon the day before the start of the intervention period, and the day after the 12-week intervention period. Serum, EDTA and Citrated blood were prepared according to protocol, and frozen. Commercially available enzyme-linked immunosorbent assays (ELISA) were used to analyse levels of vascular adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), E-selectin, P-selectin, and von Willebrand Factor (vWF). ELISA is a commonly used technique to measure the amount of an analyte in a biological sample using antibodies. Briefly, a well plate is coated with a capture antibody. The sample is added to the plate, and the target antigen is bound to the plate by the capture antibody. Then, an enzyme labelled detection antibody is added, and bind to the antigen that is already bound to the plate.

Finally, a substrate is added, that is converted into a coloured product with an optical density proportional to the concentration of the analyte [109]. L-arginine, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) levels were analysed with high-performance liquid chromatography (HPLC). In HPLC, the liquid solvent is passed through a column under pressure, which is filled with a solid adsorbent material. The molecules in the sample interacts differently with the adsorbent, causing different flow rates through the column and separation of the components. A detector monitors the absorbance or fluorescence of emerging molecules, which allows for quantitative analyses [110].

3.7 Statistical methods

3.7.1 Sample sizes

- Paper I: A previous study of patients with PAD reported a mean ±standard deviation (SD) blood velocity in the dorsalis pedis artery of 6.7 ±3.3 cm/s [95]. To detect an increase in blood velocity of 40% during INP, at least 12 subjects had to be included given 80% power and 5% significance level.
- Paper II: In a comparable population of patients with IC, pain free walking time on treadmill was 146 ±112 s [111]. Assuming an increase in pain free walking time of 87 s (76 m) as a clinically important difference [112], 26 patients per treatment arm were required to detect a treatment effect, given 80% power and 5% significance level. Assuming a withdrawal rate of 25%, we aimed to include 35 patients in each group.
- Paper III: As this was an exploratory study of secondary outcome measures, and clinically significant changes were difficult to estimate, a separate sample size calculation for this study was not performed.
- Paper IV: As this was a follow-up trial, a separate sample size calculation for this study was not performed.

3.7.2 Analyses

The statistical analyses in this thesis were performed by Henrik Hoel with support from Lars Øivind Høiseth and Jonny Hisdal. Descriptive statistics are presented as mean ±SD or median (25th, 75th percentiles) for continuous variables and number (%) for categorical variables. Normality was assessed with histograms, Q-Q plots, and residual plots. P-values < 0.05 were

considered statistically significant. Statistical analyses were performed using SPSS version 25 (IBM Statistics for Windows, IBM Corp., Armonk, NY, USA) (paper I) and Stata version 16 (Stata Inc. North Station, TX, USA) (paper II, III, IV).

In paper I, median arterial blood flow, laser Doppler flux (LDF), and MAP for each subject at every 0.5 s of the 17 s INP cycle were divided by the median values at time 0 (baseline) for the INP level being tested, giving an intra-subject relative median value for each 0.5 s of the 17 s INP cycle. The aggregated medians of blood flow and LDF for all subjects were plotted to illustrate the differences between the pressure levels (Fig 6). From the intra-subject median values, the maximal values were found for each INP level, giving the maximal blood flow, LDF and MAP relative to baseline for that subject and INP level. We used Friedman test for non-normally distributed data to examine the overall null hypothesis of no significant differences in any of the rank sums of maximal blood flow, LDF and MAP between the INP levels [113]. For pairwise comparisons of maximal blood flow and LDF between the INP levels, Dunn's post-hoc tests were performed with Bonferroni adjustment for multiple comparisons.

In paper II, differences within the groups were analysed with paired sampled t-tests. Differences between the groups were assessed with analysis of covariance (ANCOVA), adjusting for differences in baseline data [114]. All subjects with pre- and post-data available were included in the intention-to-treat analyses.

In paper III, concentrations of the measured biomarkers at baseline were plotted against concentrations after 12 weeks of treatment. The differences in baseline and post-intervention values were dichotomized to increased (≥ 0) or decreased (< 0), and differences in the distributions between the treatment group and the sham control group were compared using χ^2 test. Differences in the change of the biomarker levels between the groups were compared using ANCOVA. Differences within the groups were compared using paired sampled t-test. In the situations where data were not normally distributed, log transformations were performed. All subjects with pre- and post-data available were included in the analyses. Spearman correlation coefficients were calculated to evaluate the correlation between the change in the measured biomarkers, and the change in pain free and maximal walking distance after 12 weeks.

In paper IV, repeated measures analysis of variance (ANOVA) was performed to determine if there were changes in PWD, MWD, resting ABI and post-exercise ABI over the 24-

week treatment period. Bonferroni correction was performed for post hoc comparisons of baseline vs. 12 weeks and 12 weeks vs. 24 weeks.

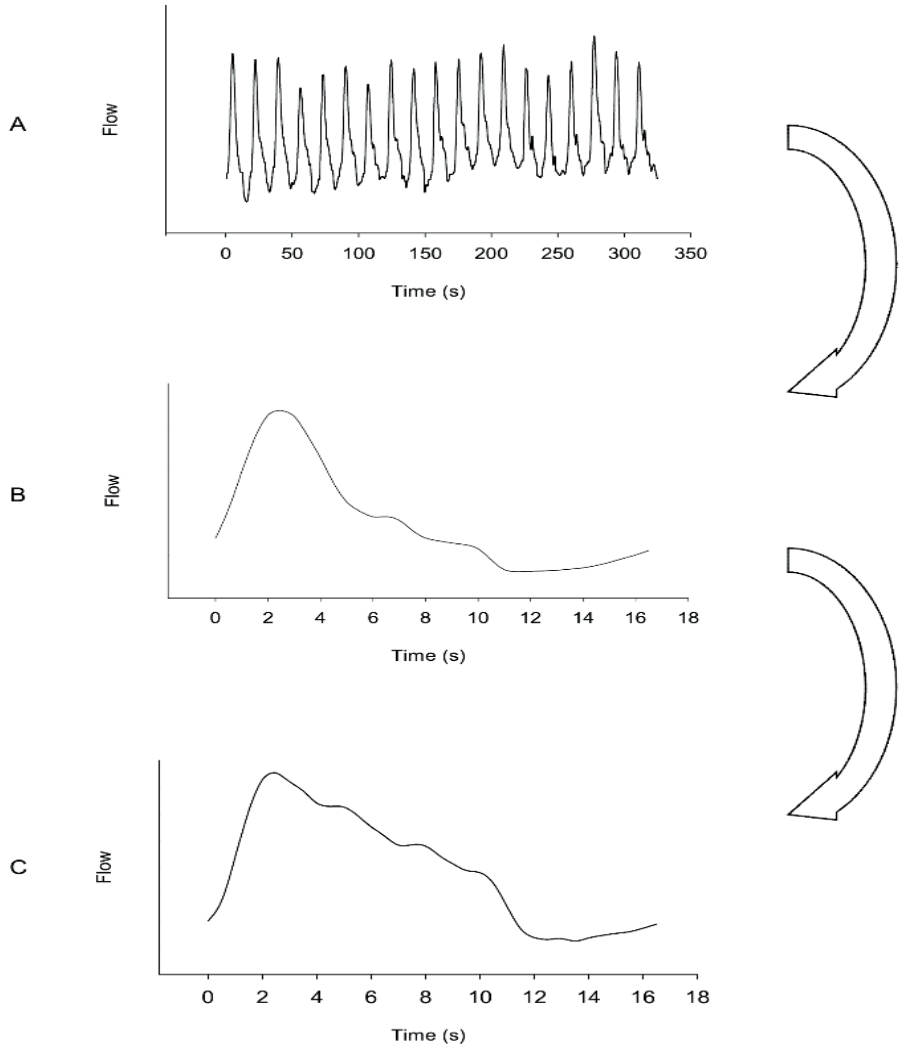

Fig 6. Illustration of how haemodynamic data was aggregated in pivot tables and plotted. Continuous measurements during application of intermittent negative pressure (A). Median values relative to baseline for every 0.5 s of the 17 s intermittent negative pressure cycle within one patient (B). Median values relative to baseline for every 0.5 s of the 17 s intermittent negative pressure cycle for all patients (C).

4. RESULTS

4.1 Paper I

In total, 16 patients with PAD were included in the study. The fluctuations in arterial blood flow and LDF through the 17 s INP cycle at each INP level are illustrated in Fig 7. At -40 mmHg INP there was an increase in maximal arterial blood flow and LDF relative to baseline of 39% and 24%, respectively. Overall, there were significant differences in maximal arterial blood flow and maximal LDF between the INP levels (both $p<0.001$). There were significantly higher maximal arterial blood flow and LDF at -40 mmHg compared with 0 mmHg ($p<0.001$ and $p=0.001$, respectively) and compared with -10 mmHg ($p=0.001$ and $p=0.025$, respectively). There were also significantly higher maximal arterial blood flow and LDF at -60 mmHg compared with 0 mmHg (both $p<0.001$) and compared with -10 mmHg ($p<0.001$ and 0.012, respectively). There were no significant differences in maximal arterial blood flow or LDF between 0 mmHg and -10 mmHg (both $p=1.0$) or between -40 mmHg and -60 mmHg (both $p=1.0$).

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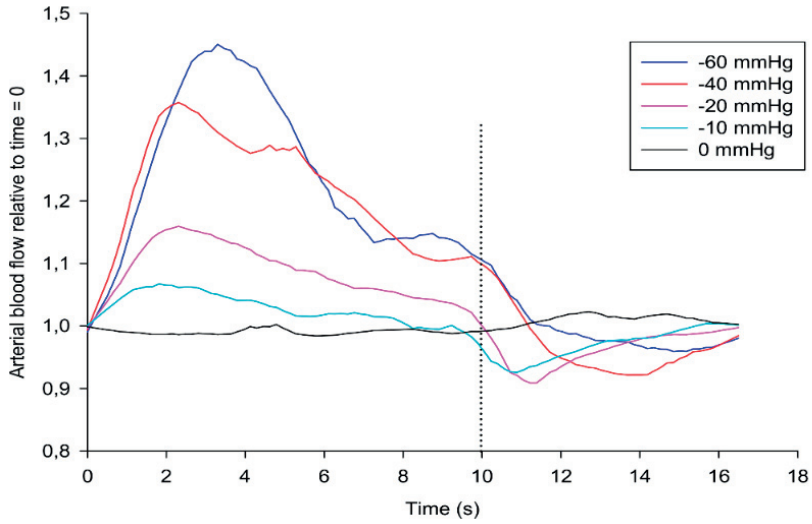

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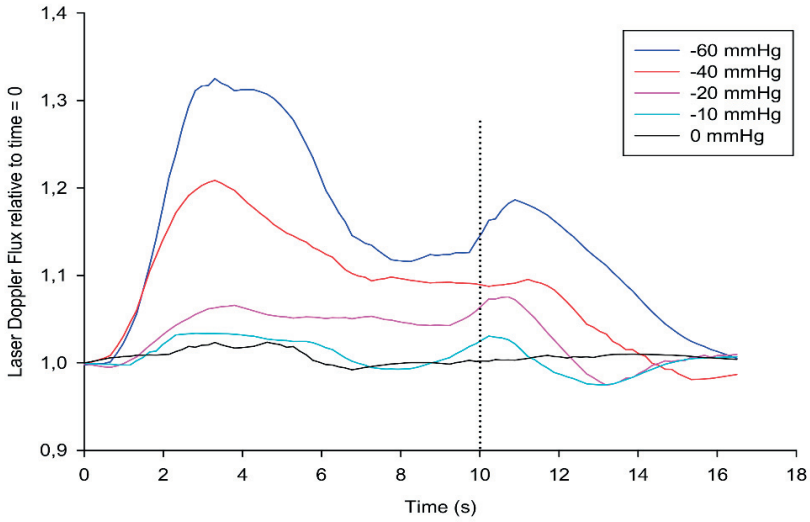

Fig 7. Arterial blood flow in the foot (A) and laser Doppler flux in acral skin of the foot (B) during the 17 s cycles of intermittent negative pressure. Aggregated medians relative to baseline (time=0 s) plotted every 0.5 s for all patients (n=16) at each pressure level. Dashed lines indicate switch from negative pressure to atmospheric pressure.

4.2 Paper II

In the RCT, 85 patients were assessed for eligibility, 72 patients were included, and 63 patients completed the 12-week intervention period. The flow of patients through the trial is illustrated in Fig 8. For all patients, between-group comparisons showed a significant difference in the change in PWD favouring the treatment group over the sham control group with an estimated treatment effect of 50 m (95% CI [11, 89]; p=0.014) after 12 weeks. There was no significant difference in the change in MWD between the two groups (estimated treatment effect 42 m (95% CI [-14, 97]; p=0.14). After 12 weeks of treatment there was an increase in PWD of 68 m (95% CI [33, 103]; p<0.001) and in MWD of 62 m (95% CI [19, 105]; p=0.006) in the treatment group and 18 m (95% CI [-1, 38]; p=0.064) and 20 m (95% CI [-16, 57; p=0.27), respectively, in the sham control group (Fig 9). Subgroup analysis of the patients classified as Fontaine IIb (baseline PWD < 200 m, n=56), showed a significant difference in the change in both PWD (estimated treatment effect 42 m; 95% [CI 2, 83]; p=0.042) and MWD (estimated treatment effect 62 m, 95% CI [5, 118]; p=0.032) favouring the treatment group over the sham control group after 12 weeks of treatment. No significant differences in the change in resting- and post exercise ABI, resting- and post ischemic calf blood flow, or the quality-of-life questionnaires EQ-5d-5l and Vascuqol-6 were shown between the groups after 12 weeks of treatment. Relative within-group changes for all outcome variables are shown in Fig 10.

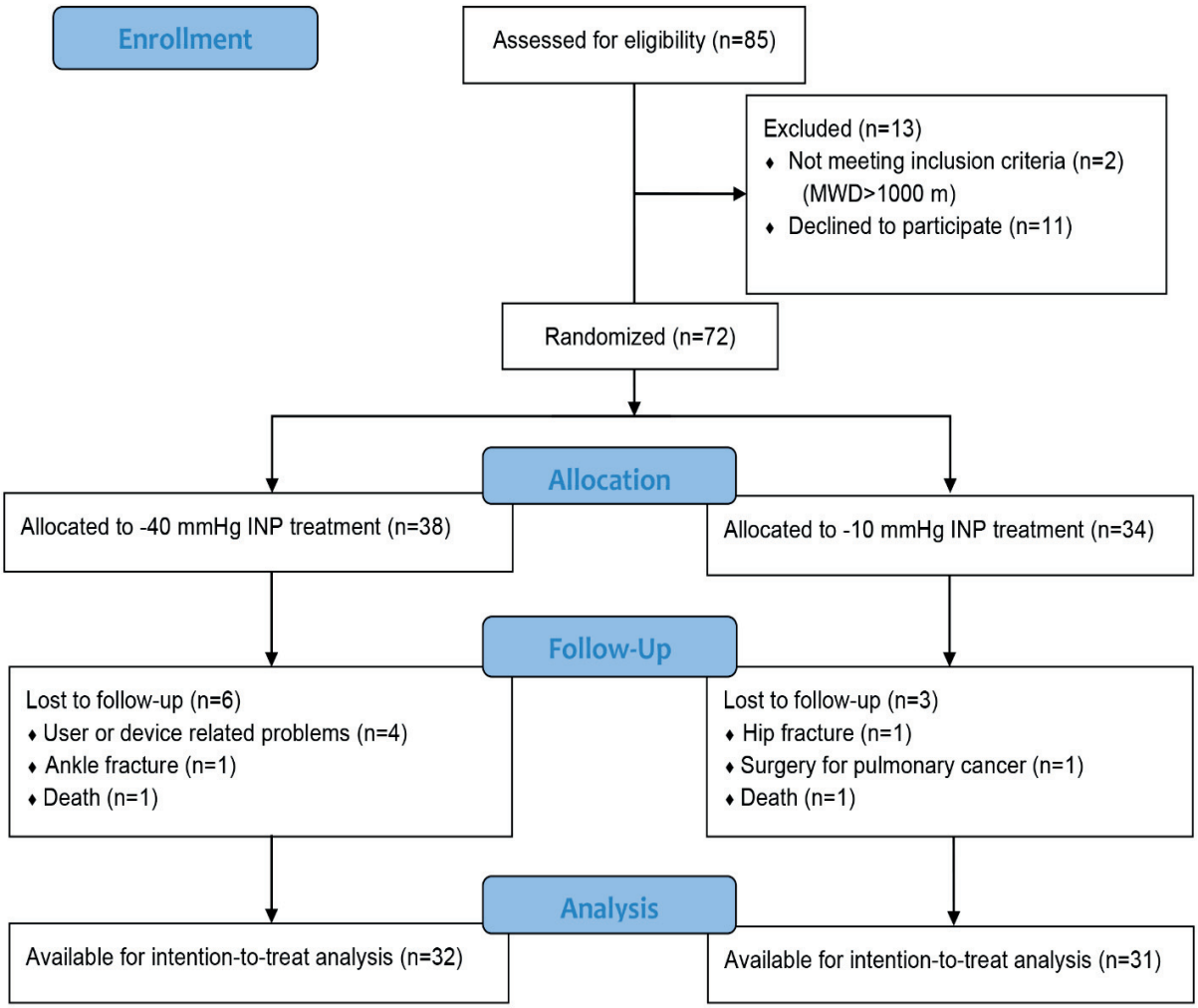

Fig 8. CONSORT flow diagram

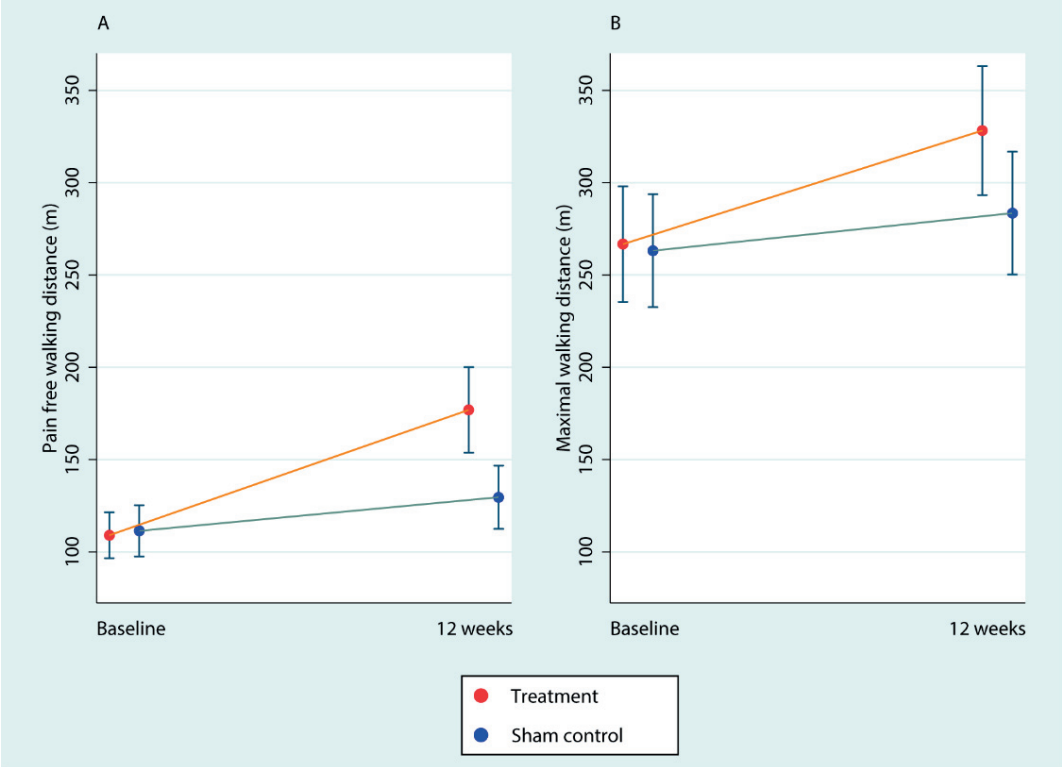

Fig 9. Pain free walking distance (A) and maximal walking distance (B) at baseline and after 12 weeks of treatment. Dots are mean values, error bars are standard errors.

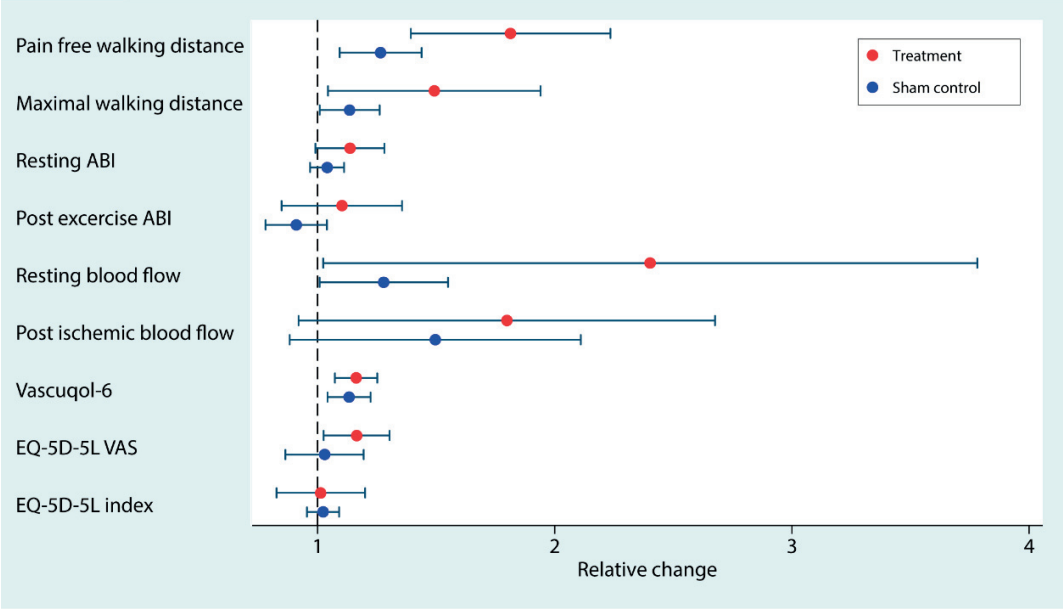

Fig 10. Relative within-group changes for all outcome variables. Dots are mean values, error bars are 95% confidence intervals.

4.3 Paper III

In total, 63 patients completed the 12-week intervention period. For all the measured biomarkers, there was a high correlation between baseline levels and levels after 12 weeks of treatment (all pairwise Spearman's rank correlation coefficients [r_s] > 0.70). Of the patients randomized to the treatment group, 25/31 (81%) had a reduction in vWF levels after 12 weeks, compared to 17/30 (57%) in the sham control group (p=0.043) (Fig 11). There were no statistically significant differences in the change in any of the biomarker levels between the groups after 12 weeks of treatment. At baseline, mean (SEM) concentration of vWF were 200% (11) in the treatment group and 189% (9) in the sham control group. Within the treatment group there was a significant reduction in the concentration of vWF of -11% (4) (p=0.019), whereas there was no significant change in the levels of vWF in the sham control group (1% (6); p=0.85). For all the other measured biomarkers, no significant changes were shown. There was no significant correlation between the change in vWF and the change in PWD (r_s=-0.22, p=0.088), and no significant correlation between the change in vWF and the change in MWD (r_s=-0.07, p=0.61) after 12 weeks.

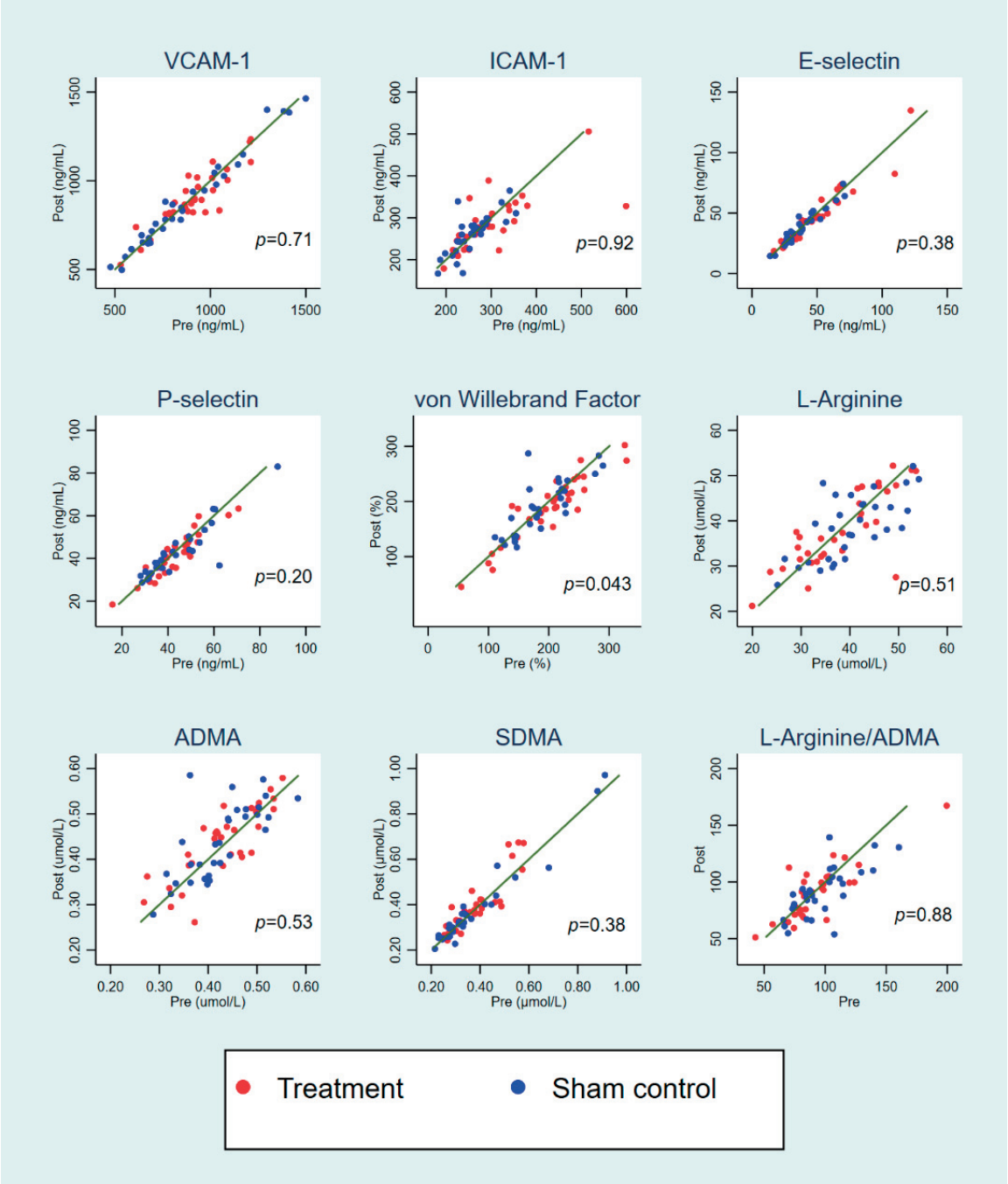

Fig 11. Concentrations of vascular biomarkers at baseline and after 12 weeks of intermittent negative pressure treatment. Reference lines indicating post values = pre values. P-values refer to χ^2 tests of proportions of patients with values increased versus decreased after 12 weeks. VCAM-1 vascular adhesion molecule-1; ICAM-1 intracellular adhesion molecule-1; ADMA asymmetric dimethylarginine; SDMA symmetric dimethylarginine.

4.4 Paper IV

Of the 32 patients who completed 12 weeks of active treatment with -40 mmHg INP in the RCT, 10 patients (31%) volunteered to continue treatment for 12 additional weeks (24 weeks in total) and were included in the follow-up trial. At baseline, mean ±SD PWD was151 ±91 m. A repeated measures ANOVA showed that 24 weeks of INP treatment had a statistically significant effect on PWD ($F(2,18)=6.95$; $p=0.006$) (Fig 12). Post hoc tests revealed that PWD increased significantly from baseline to 12 weeks (mean 81 m; 95% CI [6, 156]; $p=0.032$), but there was no significant change in PWD from 12 to 24 weeks (mean 19 m; 95% CI [-56, 94]; $p=1.00$). At baseline, MWD was 362 ±159 m. For MWD, a repeated measures ANOVA showed that 24 weeks of INP treatment had a statistically significant effect on MWD ($F(1.198,10.780)=8.55$; $p=0.012$) (Fig 12). Post hoc tests showed no significant change in MWD from baseline to 12 weeks (mean 38 m; 95% CI [-85, 161]; $p=1.00$), but a significant increase from 12 to 24 weeks (mean 145 m; 95% CI [22, 268]; $p=0.018$). At baseline, resting ABI was 0.53 ±0.12, and post exercise ABI was 0.28 ±0.12. There were no significant effects of INP treatment on resting ABI or post exercise ABI during the 24-week treatment period ($F(2,18)=2.06$; $p=0.157$ and $F(2,11)=0.86$; $p=0.450$, respectively).

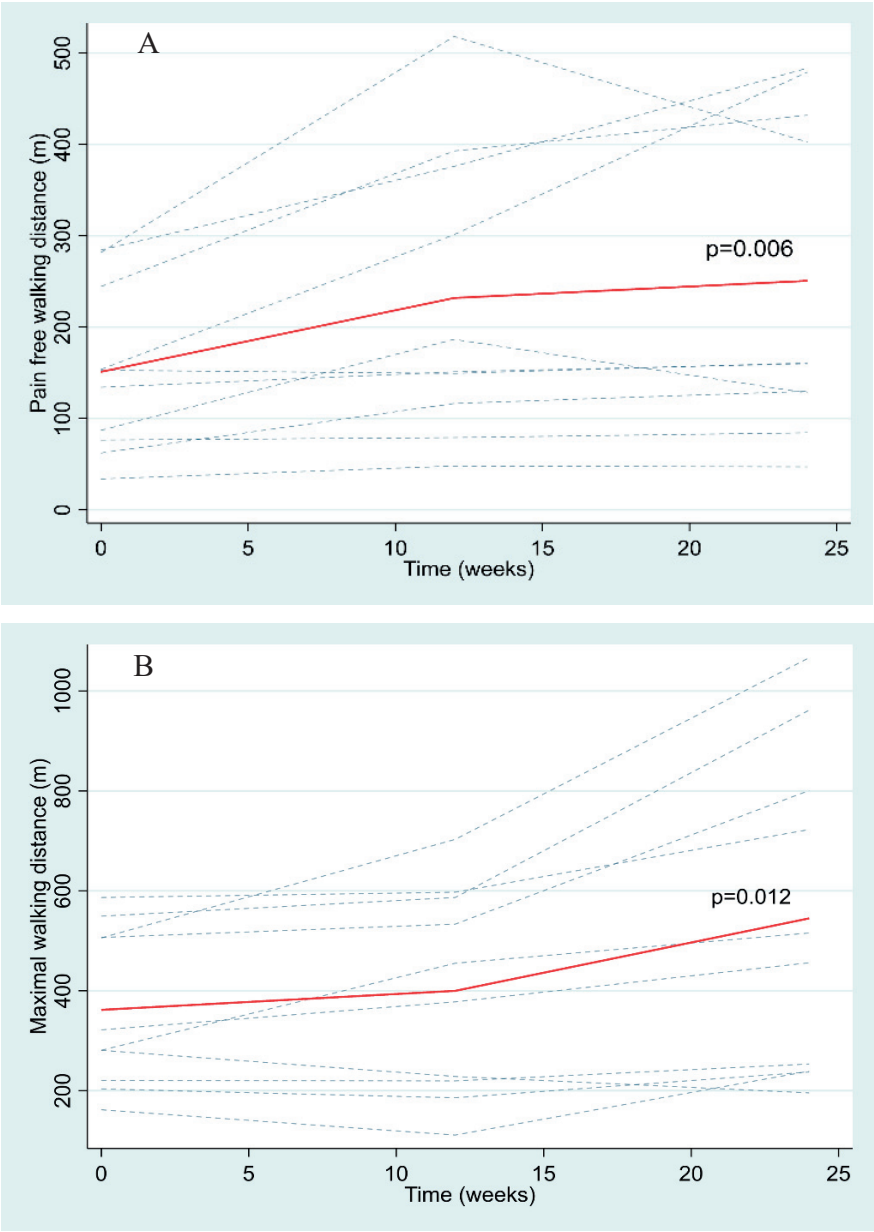

Fig 12. Pain free walking distance (A) and maximal walking distance (B) plotted at baseline, 12 weeks, and 24 weeks of treatment with lower extremity intermittent negative pressure. Blue dashed lines represent individual patients (n=10), red lines represent the mean values at each time point. Overall P-values for repeated measures ANOVA are presented.

5. DISCUSSION OF MAIN FINDINGS

5.1 Acute effects of intermittent negative pressure

In paper I, we found that -40 mmHg and -60 mmHg INP increased arterial and skin blood flow in patients with PAD. There was no significant difference in maximal arterial and skin blood flow between -60 mmHg and -40 mmHg, hence -40 mmHg was the lowest level of INP that induced such changes. An INP level of -10 mmHg did not significantly induce an increase in maximal arterial and skin blood flow compared to atmospheric pressure alone.

An INP level of -40 mmHg has been suggested to be effective to acutely increase blood flow in the lower extremities in previous studies [94, 95, 97]. However, older studies of the effects of INP treatment used INP levels of -120 mmHg, -150 mmHg and -200 mmHg [86, 89-91, 115]. Hence, the level of INP, but also the treatment frequency, the way in which negative pressure was applied, and the length of negative pressure periods and atmospheric pressure periods varies in the literature. The theoretical rationale of applying INP instead of constant negative pressure is to avoid the effect of the veno-arterial reflex or the postural vasoconstrictor response in the leg, which causes vasoconstriction of arterioles when the veins become distended [116]. Activation of this reflex explains why the acute increase in flow rate during onset of negative pressure is followed by a decrease in the flow rate [94]. In healthy subjects, the veno-arterial reflex counteracts haemodynamic changes when moving from supine to standing position, and by increasing precapillary resistance, limits a rise in hydrostatic pressure and prevents oedema. In patients with PAD, this reflex seems to be reduced or absent, but reappear after revascularization [117]. The reflex is dependent on a local neurogenic mechanism, with the contribution from a local myogenic response, and a centrally elicited sympathetic component [118]. When INP is applied to the lower leg, the activation of the veno-arterial reflex is probably dependent on the level of negative pressure, but also for how long that pressure level is applied before returning to atmospheric pressure. Hence, the optimal level of INP to improve blood flow is dependent on the length of the negative pressure period and the length of the atmospheric pressure period during the INP cycle. Consequently, higher INP levels should be applied in shorter pulses than lower INP levels.

An INP level of -10 mmHg did not significantly affect maximal arterial and skin blood flow. Otherwise, application of -10 mmHg INP appeared identical to -40 mmHg, and the difference between -10 mmHg and -40 mmHg was impossible to distinguish for the research

subjects without any reference, hence -10 mmHg seemed to be the proper INP level for a sham device.

5.1.1 Effects of intermittent negative pressure on central hemodynamic

Theoretically, exposure of the lower limb to INP, could affect systemic arterial pressure. In paper I, we recorded MAP during application of INP, showing that there were no significant differences in the maximal change in MAP for INP levels up -60 mmHg (Fig 13). Hence, a change in MAP does not explain the changes in flow rate in the leg during INP. Further, this indicate that INP treatment seems safe for patients with cardiovascular comorbidities, potentially sensitive to rapid hemodynamic changes.

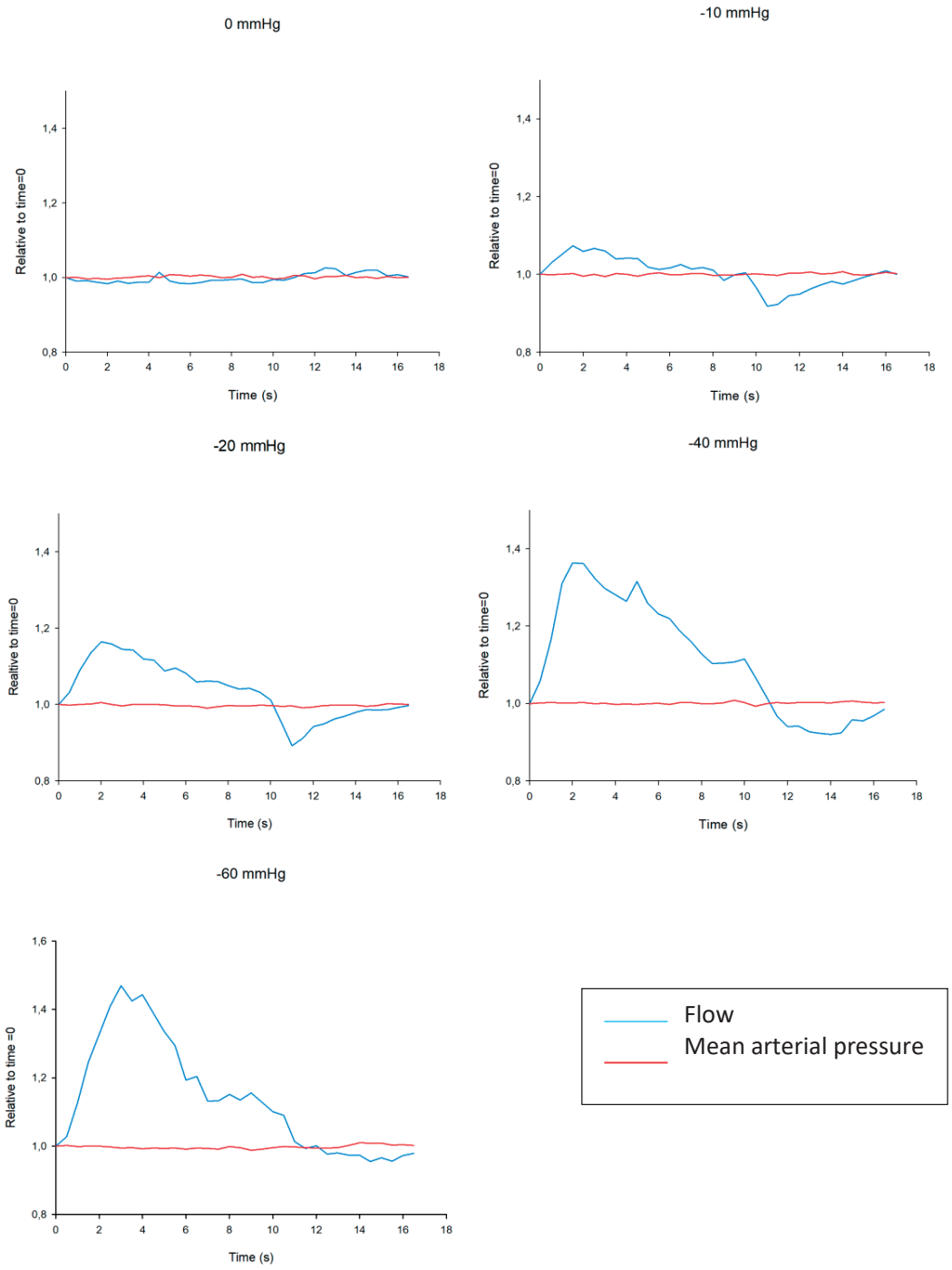

Fig 13. Aggregated medians of arterial blood flow in the dorsalis pedis or tibialis posterior artery and systemic mean arterial pressure for every 0.5 s of the 17 s INP cycle.

5.1.2 How does intermittent negative pressure acutely increase blood flow?

The physical explanation for the acute increase in flow rate during INP can be derived from fluid mechanics. Poiseuille's law for laminar flow of an incompressible Newtonian fluid through a cylindric tube, states that the flow rate is proportional to the pressure difference between the two sides of the tube and the radius of the tube, and inversely proportional to the length of the tube, and the fluid viscosity [119].

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

Q: flow rate, ΔP: pressure difference between the two ends of the tube, L: length of the tube, μ: fluid viscosity, r: radius of the tube.

In 1968, Caro et al. observed that when the extremity was exposed to a short period of negative pressure inside a pressure chamber, the venous pressure followed the magnitude of the pressure applied in the pressure chamber [120]. The negative pressure phase of the INP cycle has very little effect on the arterial pressure, but causes a drop in venous pressure, resulting in an increased arteriovenous pressure difference with a magnitude similar to the level of the negative pressure applied [89]. The different impact of INP on the arterial and venous side is explained by different anatomical and physiological properties: the arteries have relatively high intraluminal pressure and thick walls, whereas veins have relatively low intraluminal pressure, thin walls, and valves. The increased arteriovenous pressure difference generated during the onset of negative pressure leads to increased arterial flow, which then gradually decreases as the arteriovenous pressure difference becomes equalized. The switch to atmospheric pressure promotes venous outflow, which avoids the effects of the veno-arterial reflex before the next INP cycle is initiated.

5.2 Long term effects of intermittent negative pressure treatment

5.2.1 Walking distance

The main finding in paper II was that treatment with -40 mmHg INP twice daily for 12 weeks increased PWD compared to sham treatment in patients with IC. For patients with baseline PWD < 200 m (classified as Fontaine IIb), there was an increase in both PWD and MWD.

The positive effect of INP treatment on walking distances in patients with IC are in line with several previous studies [89-91]. Recently, Hageman et al. and Afzelius et al. concluded in two RCTs, that INP treatment did not have an additional effect to SET or home-based physical activity and life-style changes in increasing walking capacity in patients with IC [99, 100]. An explanation for these findings might be that the effect of increased physical activity surpasses a potential effect of INP treatment. Multiple studies have documented the beneficial effects of SET programs on walking capacity, functional status, and quality of life in patients with IC [67, 68, 70-72, 121]. However, a systematic review from 2016 concluded that only one third of the patients with IC were suitable for and willing to undertake SET, and outlines that the adherence to SET programs are poor [75]. Despite that SET should be the first choice of treatment for patients with IC, INP treatment may be a useful supplement when SET is unavailable, or for patients who are unable or unwilling to participate in SET. Another possible explanation for the contradictory results in the studies by Hageman et al. and Afzelius et al. is that the patients were treated with INP only for 30 and 40 minutes two and three times a week for six weeks, respectively, which are significantly shorter treatment times and treatment frequencies compared to our study. The reason is probably that the INP system used by Hageman et al. and Afzelius et al. was dependent on in-hospital treatment instead of home treatment. As INP applied to the lower leg induces acute rhythmical fluctuations in blood flow [94, 95, 122] that may promote the long-term favourable effects leading to increased walking capacity, it is reasonable to assume that a higher treatment frequency might be necessary to achieve clinical effects.

In paper II, we found a significant treatment effect of INP treatment on PWD of 50 m (95% CI [11, 89]) compared to sham treatment for all patients, and an effect of 42 m (95% CI [2, 83]) for PWD and 62 m (95% CI [5, 118] for MWD for patients classified as Fontaine IIb. The minimal clinically important improvement in walking distance in patients with IC after interventions have been suggested [112], however as this is related to the severity of the disease, comorbidities, and the patient's subjective judgement, no consensus is established. Pharmacological agents such as cilostazol and pentoxifylline have market approval in Europe and the US, with the indication of improving leg function in patients with IC, however, are not routinely recommended for PAD patients in Europe [36]. A Cochrane review from 2014 estimated that cilostazol may increase PWD by 31 m (95% CI [22, 40]) and MWD by 43 m (95% CI [18, 68]) compared to placebo [123], and another Cochrane review from 2015 looking at the clinical

effects of pentoxifylline in patients with PAD found an improvement in PWD of -33.8% to 73.9% and in MWD of 1.2% to 156%, but statistical tests were not performed due to insufficient data [124]. Hence, the effect estimates of INP treatment in our study seems to be competitive to the effect estimates for cilostazol and pentoxifylline treatment in improving walking distance in patients with IC.

In paper IV, we found that for the 10 patients who volunteered to continue active treatment with -40 mmHg INP for additional 12 weeks (24 weeks in total), there was an increase in both PWD and MWD after 24 weeks, compared with baseline. The main increase in PWD occurred during the first 12 weeks of treatment, whereas the main increase in MWD occurred from 12 to 24 weeks of treatment. This may be explained by the fact that many patients with IC have a low exercise capacity due to concomitant heart and lung diseases, which in addition to the leg pain, may restrict their MWD. An initial improvement in PWD may allow for more physical activity, which in turn improves the exercise capacity and the MWD. As we did not monitor the patients' activity level during the 24-week treatment period, we cannot adjust for a possible impact of increased physical activity on the walking distances in the follow-up trial. The patients' reasons for why they wanted to continue the treatment were also not quired. It may have been related to an improvement for the first 12 weeks and an expectation to continue the improvements for the coming 12 weeks, or it may be because they did not improve during the first 12 weeks and hoped for an improvement during the additional 12 weeks, or it may not have been related to the treatment effect for the first 12 weeks. Hence, there is a potential risk of selection bias in the follow-up trial. To illustrate the sample of patients included in the follow-up trial, their PWDs and MWDs are plotted together with the PWDs and MWDs for all the other patients in the active treatment group who only received treatment for 12 weeks in Fig 14.

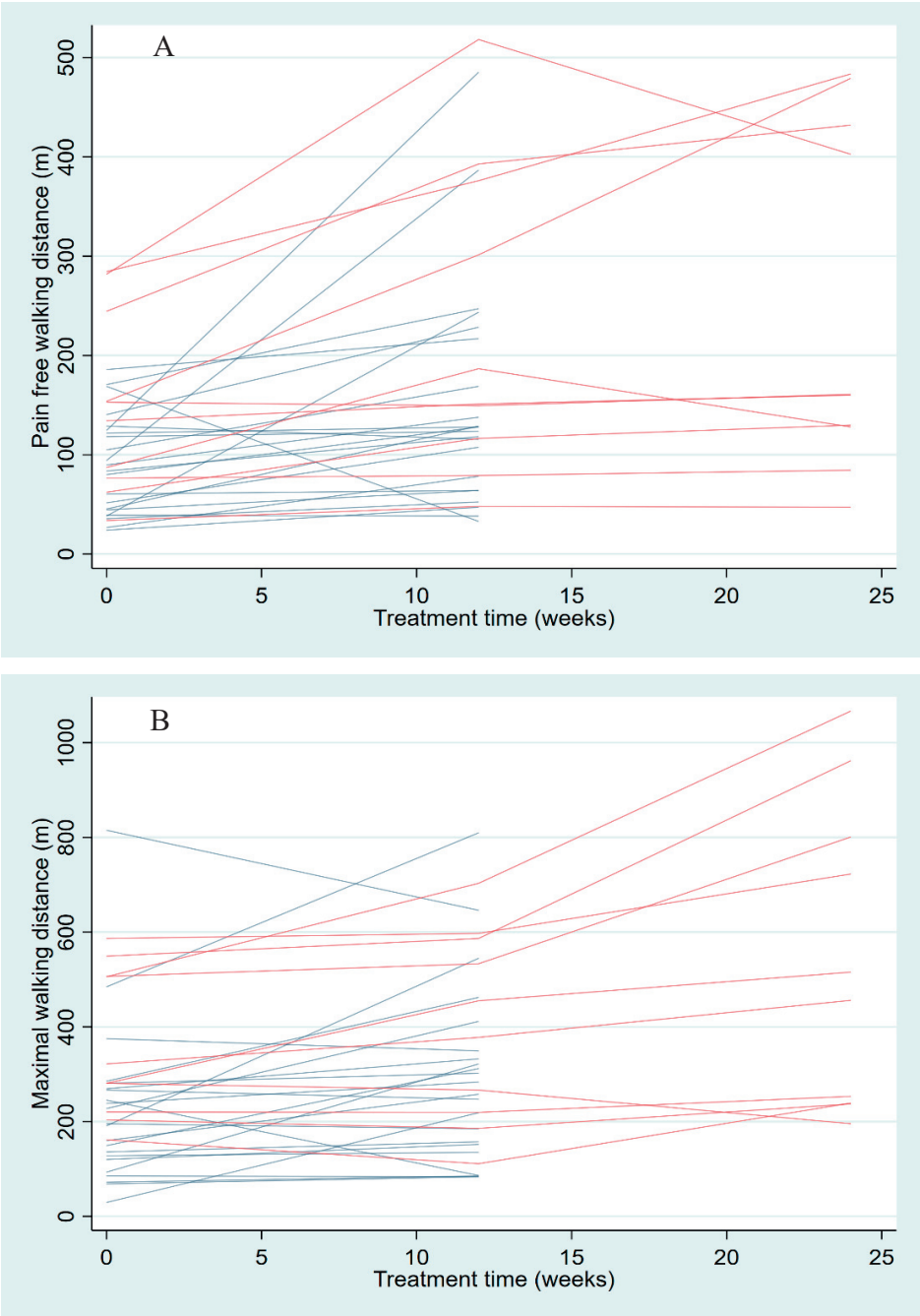

Fig 14. Pain free walking distance (A) and maximal walking distance (B) plotted at baseline, 12 weeks, and 24 weeks of treatment with lower extremity intermittent negative pressure. Blue lines represent patients receiving treatment with -40 mmHg intermittent negative pressure for 12 weeks, red lines represent patients receiving treatment with -40 mmHg intermittent negative pressure for 24 weeks.

5.2.2 Quality of life, ankle-brachial index, and calf blood flow

In paper II, we did not find a significant difference in the change of EQ-5d-5l or Vascuqol-6 between the active treatment group and the sham control group after 12 weeks of INP treatment. This is contradictory to findings in studies on the effects of SET for patients with IC [71, 72]. A possible explanation is that the improvement in quality of life observed after SET is related to more than the improvement in walking distance, such as increased physical activity and social interactions, which is not obtained in the same way by INP treatment alone. It is also possible that our study was underpowered to detect quality of life changes across the two groups. However, direct comparison of the treatment effects presented in paper II with studies on SET is methodologically problematic as studies on SET cannot be double blinded.

We did not find any differences in the changes of ABI or resting- or post ischemic calf blood flow between the groups after 12 weeks of treatment. A systematic review of the effects of exercise training in patients with IC did also conclude with an effect on walking capacity without any effect on ABI [70]. Contradictory to our calf blood flow findings, Hiatt et al. did find a moderate increase in maximal calf blood flow after exercise training, but the increase did, however, not correlate with the improvement in exercise performance [72].

5.2.3 Circulating vascular biomarkers and endothelial function

In paper III, we found that a significantly higher proportion of the patients receiving treatment with -40 mmHg INP twice daily for 12 weeks had a reduction in vWF, compared with the patients receiving sham treatment. Further, we observed a significant reduction in the plasma concentration of vWF within the treatment group after 12 weeks. These findings might indicate a beneficial effect of INP treatment on endothelial activation and endothelial injury, even though no differences in the change in the absolute levels of vWF between the groups were observed. No significant changes were observed in the levels of the adhesion molecules VCAM-1, ICAM-1, E-selectin and P-selectin, or the nitrogen monoxide (NO) related molecules L-arginine, ADMA and SDMA after 12 weeks of INP treatment.

vWF is a glycoprotein synthesized and stored in endothelial cells and plays important roles in primary haemostasis by mediating platelet adhesion and aggregation to sites of endothelial injury, and also mediates coagulation by stabilizing coagulation factor VIII in the circulation [125]. Circulating levels of vWF are increased in patients with PAD [126], and are

suggested to have a prognostic value for patency after infra-inguinal bypass grafting, and for future risk of cardiovascular events [127, 128]. Although based on proportion calculations and within-group comparisons, the observed reduction in vWF after INP treatment suggests that INP treatment could reduce prothrombotic endothelial properties in PAD patients.

In a previous study, Sundby et al. demonstrated that flow rate also was increased in a 5-minute period after the INP treatment was ended [95]. Rhythmic changes in flow rate leads to increased shear stress between the blood and the endothelium of the arterial wall, which promotes local release of NO followed by vasodilation [129], a physiological phenomenon known as endothelial dependent or flow-mediated dilation. A recent study investigating effects of INP on flow-mediated dilation, concluded that fluctuations in shear stress pattern during INP, improved brachial artery flow-mediated dilation, and suggested that fluctuations in blood flow represent a stimulus that acutely affect endothelial function [130]. Impaired vasodilator function has been demonstrated in patients with PAD [131], and impairment of the NO synthesis pathway may be one explanation for the limited exercise capacity in patients with IC [67]. NO is a potent vasodilator with a half-life of seconds in the blood. It is produced in the endothelial cells by the enzymatic conversion of L-arginine mediated by nitric oxide synthase, and rapidly diffuses over cell membranes to the underlying smooth muscle cells, and turn off their contraction, resulting in dilation of the arteries. Endothelium-derived NO plays important roles in vascular homeostasis through antiatherogenic and antiproliferative effects on the arterial wall, and also exert angiogenic effects [132]. ADMA and SDMA are endogenous products of proteolysis, which inhibit NO synthesis. ADMA inhibits NO synthase by competing with L-arginine on the active site of the enzyme, while SDMA inhibits the cellular uptake of the NO precursor homoarginine. ADMA and SDMA are sensitive markers for endothelial dysfunction, and homoarginine/ADMA ratio and homoarginine/SDMA ratio are independent predictors for long-term cardiovascular mortality and events in patients with lower extremity PAD [133]. One study reported an increase in flow-mediated vasodilation in the brachial artery of 61% after 6 months of aerobic exercise rehabilitation in patients with IC [134]. As some of the local physiological effects of INP treatment may be comparable to the local effects of physical exercise, it might be that the improvement in walking capacity after INP treatment is related to improvement in flow-mediated vasodilation (Fig 15). We did however not find any effects on circulating levels of L-arginine, ADMA or SDMA after 12 weeks of INP treatment, which may indicate that despite improving walking

capacity in patients with IC, INP treatment does not seem to affect the NO synthesis pathway measured at a systemic level.

Upregulation of adhesion molecules in response to non-laminar blood flow or other proinflammatory stimuli are an important feature of the atherosclerotic process [15], hence circulating levels of soluble adhesion molecules such as ICAM-1, VCAM-1, E-selectin, and P-selectin may reflect the inflammatory response of the endothelium. We did not observe changes in circulating levels of VCAM-1, ICAM-1, E-selectin, and P-selectin after 12 weeks of INP treatment. This does not correspond to findings in a study of the effects of SET in patients with IC, which reported a significant reduction in E-selectin and ICAM-1 after 8 weeks, and indicate that INP treatment of one leg does not affect the total vascular inflammatory burden caused by atherosclerosis, in contrast to what is observed after a period with SET in patients with IC.

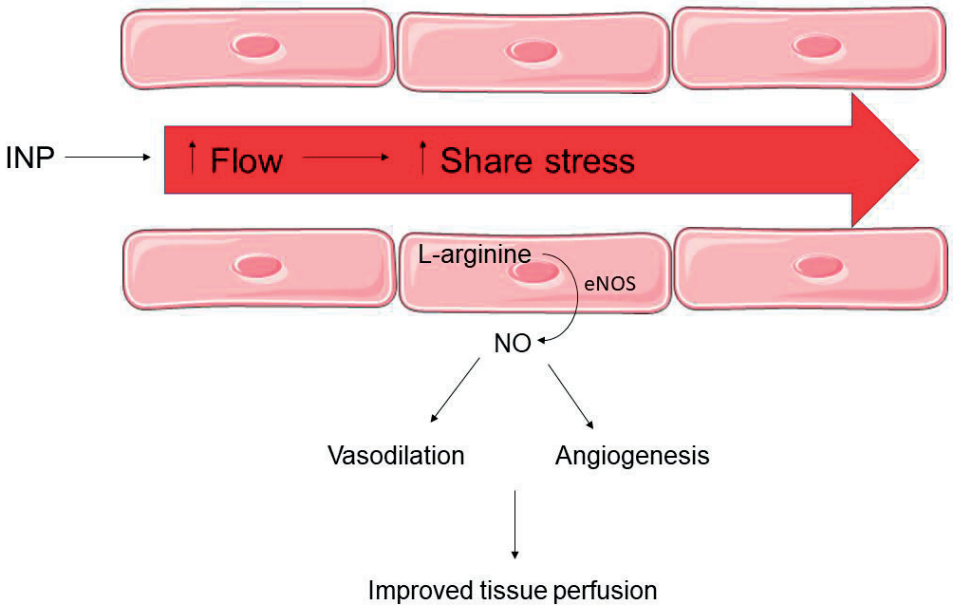

Fig 15. Model of how intermittent negative pressure treatment may improve tissue perfusion by promoting flow-mediated dilation and angiogenesis. eNOS endothelial nitric oxide synthase; NO nitrogen monoxide. The figure is created by Henrik Hoel with elements adapted from Servier Medical Art: smart.servier.com.

6. METHODOLOGICAL CONSIDERATIONS

6.1 Measurements of arterial and skin blood flow

The Doppler effect is the phenomenon that the frequency of a wave changes if either the source or the observer is moving [135], and is valid for all electromagnetic and acoustic waves. In ultrasound Doppler, ultrasound with a predefined frequency is transmitted to the moving blood inside a blood vessel. The soundwave is reflected by the moving blood, and the shift in frequency between the transmitted and reflected sound is recorded. The frequency shift is dependent on two factors, blood flow direction and velocity. To calculate blood flow, the cross-sectional area of the blood vessel must be measured simultaneously. In the experimental setup described in paper I, it was not possible to measure the vessel diameter inside the pressure chamber during INP. Therefore, the diameters of the dorsalis pedis artery and tibialis posterior artery were measured with an ultrasound triplex scanner before application of INP, and arterial blood velocity was measured with the pulsed ultrasound Doppler probe fixed to the foot during application of INP. Hence, any change in vessel diameter during application of INP was not recorded and may have resulted in inaccurate estimates of blood flow. As flow-mediated dilation may occur, there might have been an underestimation of arterial blood flow during INP. The pulsed ultrasound Doppler probe was fixed to the foot over the dorsalis pedis- or tibialis posterior artery, at the place where the best Doppler signal was achieved. Hence, small displacements of the probe during the experiments may also have caused an underestimation of the arterial blood flow measurements.

In laser Doppler flowmetry the laser light penetrates the tissue to a depth dependent on the light frequency, and the light is reflected from immobile or moving tissues. The proportion of the light that is reflected from moving blood cells goes through a Doppler shift, and by sampling all reflected light, the device can calculate the flux of erythrocytes within the sample volume. The penetration depth is usually around 1 mm, hence includes capillaries in the skin [136]. We measured the LDF inside the pressure chamber during application of INP with a laser Doppler probe attached to the pulp of the first toe. As the probe measures LDF at a depth of 1 mm, pressure on the probe or small displacements may have resulted in inaccurate measurements. During the 17 s INP cycle, we observed an increase in LDF during the 10 s negative pressure, with a peak after 3 s, but we did also observe a second peak around 11 s, after return to atmospheric pressure (Fig 7 B). The reason for this second peak might be explained by the fact

that laser Doppler flowmetry does not measure the direction of flow, hence the second peak might be caused by a backward movement of the red blood cells within the capillaries.

6.2 The randomized controlled trail

The RCT has emerged as the gold standard design for clinical trials since the evaluation of streptomycin for the treatment pulmonary tuberculosis by the British Medical Research Council in 1948 [137]. The purpose of randomization is to obtain that known and unknown factors that potentially can affect the course of the disease become randomly distributed between the groups. Blinding is necessary to make sure that subjective assumptions of the treatment effect by the research subject or the researcher does not affect interpretations and measurements [138]. Hence, if possible, a RCT should be blinded to the research subject, and the researcher. In our RCT, patients were randomized to active treatment with -40 mmHg INP or sham treatment with -10 mmHg INP. It was not possible for the clinical personnel participating in the data collection or patient follow-up to distinguish between the two pressure levels. For the patients, who had no reference of what to feel during INP, it was also not possible to distinguish between the two pressure levels. An important feature of RCTs is to prevent selection bias using concealed allocation, meaning that the person assigning the patients to the groups does not know in advance what the next group allocation will be. This is to prevent that prognostic factors affects which patients are assigned to which group [139]. In our RCT, labelling of the treatment devices were performed by a person at the company producing the INP device (Otivio AS), and individuals involved in patient enrolment, data collection and laboratory analyses were blinded to the group allocation.

6.2.1 Internal and external validity

The internal validity of a RCT is determined by how well the study can rule out alternative explanations for its findings. That is why RCTs have a strict predefined design with inclusion- and exclusion criteria, and should be reported according to the CONSORT statement [101]. The strict design reduces the risk of systematic errors or bias, and is the key advantage of RCTs. On the other hand, to be clinically useful, the results must be relevant to groups of patients in a clinical setting. This is often referred to as the generalisability, or the study's external validity. Lack of consideration of external validity is a common criticism of RCTs by clinicians [140].

In our RCT, participation in SET was not an inclusion or exclusion criterion, but all patients were given the same information recommending physical exercise. This contrasts to the studies by Hageman et al. and Afzelius et al., where all study subjects had to agree to participate in SET or homebased exercise therapy to be eligible for participation in those two studies, respectively. A requirement of participation in physical exercise in order to be included in the study may increase the internal validity. Further, participation in SET comply with the current guidelines on the treatment of IC [36]. However, as literature shows that only 1 in 3 patients with IC are suitable for and willing to undertake SET [74], participation in SET does not reflect the large population of IC patients, hence the external validity of the results from those two studies seems questionable. In our RCT, there may have been differences in physical exercise levels between the treatment group and the sham control group, however, as the patients were randomized, we would expect this to be similar at baseline. It might be that the effect of INP treatment in the treatment group, causes the patients to increase their physical activity, which in turn may have affected the estimated treatment effect after 12 weeks. As we did not measure the patients' activity levels during the intervention period, this potential synergistic effect on walking distance could not be adjusted for in our analyses.

6.3 Active treatment versus sham control

In paper I, we concluded that the maximal increase in arterial and skin blood flow at -10 mmHg INP were statistically not different from atmospheric pressure, and hence an appropriate INP level for a sham device. However, the plots of arterial and skin blood flow during the INP cycle (Fig 7), indicate that there might be a small acute effect on arterial and skin blood flow at -10 mmHg, even though statistically not significant. Hence, we cannot rule out that the repetitive exposure to -10 mmHg INP over time could have had an impact on the walking capacity in the sham control group, which may have resulted in an underestimation of the treatment effect. However, the use of -10 mmHg INP in the sham device was necessary to achieve true blinding of the patients and the clinical personnel. To validate if true blinding was achieved, patients could have been queried after the treatment period which INP level they thought they received. Unfortunately, that question was not included in our follow-up questionnaire, however, at least two findings indicate true blinding of the patients. Firstly, compliance (mean daily treatment time) was similar in the treatment group and the sham control group. In the event patients

believed they were in the sham control group; we would expect their motivation to sit for two hours per day would be reduced and compliance would be lower. Secondly, more patients were lost to follow-up in the treatment group than in the sham control group. In the case true blinding was not achieved we would have expected a higher drop-out rate in the sham control group than in the treatment group.

6.4 Treatment of one leg

In the RCT, patients with bilateral IC symptoms were instructed to treat only the most symptomatic leg reported after the baseline treadmill test. For six patients in the active treatment group, and four patients in the sham control group, MWD was limited by the opposite leg at the 12-week follow up. This was not possible to adjust for in the statistical analyses, but may have affected the estimated treatment effect. Ideally, all patients should have treated both legs at the same time throughout the treatment period. The FlowOx 2.0 system is designed for the use of two pressure chambers connected to one control unit. However, regulatory wise, the use of two pressure chambers per control unit has not been approved, hence was not available at the time of our study.

6.5 Walking distance as the primary endpoint

The primary endpoint in paper II and IV was change in PWD and MWD measured on treadmill using the Gardner-Skinner progressive protocol, starting with a constant speed of 3.2 km/h, at 0% slope, increasing the slope by 2% every 2 minutes [102]. Other walking tests such as the constant load treadmill tests and the 6-minute walk test are also frequently used for assessment of walking capacity in patients with PAD, and which test is the most reliable is debated [141]. A study by Gardner and Skinner from the early 1990 concluded that a progressive stage treadmill test better assessed walking capacity in patients with PAD than the single stage treadmill test [102]. However, a recent metanalysis of four RCTs, comparing 6-minute walk test with treadmill tests, found that patients randomized to the control groups receiving no treatment or placebo treatment improved their treadmill MWD, but declined their 6-minute walking distance during the study period, and concluded that the treadmill walking outcome does not detect the functional decline over time as the 6-minute walk test, which limits the usefulness of treadmill testing as a meaningful outcome measure for people with PAD [142]. Notably, we observed a mean increase of

20 m on MWD in the sham control group. Hence, the improvements observed in the sham control group might have been less pronounced using 6-minute walk test to measure the primary outcome instead of the progressive stage treadmill test. Ideally, both progressive stage treadmill test and 6-minute walk test should have been performed.

6.6 Calf blood flow measurements

Strain-gauge plethysmography is a non-invasive technique used for measurements of limb blood flow, and by some considered as the gold standard analysis of peripheral blood flow in patients with PAD [143]. However, the method does have issues regarding reproducibility [105]. We did not find a difference in the change of resting- or post-ischemic calf blood flow measured by strain-gauge plethysmography between the treatment group and the sham control group after 12 weeks of treatment. As illustrated in Fig 10, the confidence intervals around the mean values were wide, probably caused by poor reproducibility, even though the measurements were performed by the same person at baseline and after 12 weeks of treatment. One explanation might be that the strain-gauge was not placed at the exact same point around the leg, or with a different angle around the leg at each measurement. We thought that the calf blood flow measurements could add additional information explaining a potential treatment effect after INP treatment. However, as the strict study design of an RCT gives very limited opportunities to sort out poor or obviously incorrect measurements, this was not the case.

6.7 Quantification of circulating vascular biomarkers

In the RCT (paper III), venous blood samples were collected from the patients at baseline and after 12 weeks of treatment, to measure changes in circulating vascular biomarkers. We found that a significantly higher proportion of the patients receiving treatment with -40 mmHg INP twice daily for 12 weeks had a reduction in vWF, compared to the patients receiving sham treatment. Further, there was a significant reduction in the plasma concentration of vWF within the treatment group after 12 weeks, however no differences between the groups were observed. For VCAM-1, ICAM-1, E-selectin, P-selectin, L-arginine, ADMA and SDMA no significant changes were observed after 12 weeks of INP treatment. This was an exploratory study of secondary outcome measures, and a separate sample size calculation were not performed. Hence, the study may have been underpowered to detect significant between-group differences. The

patients were instructed to treat only their most limiting leg throughout the 12-week period. As atherosclerosis is a systemic disease, INP treatment of one leg may not have been sufficient to affect circulating levels of the measured biomarkers enough to show between-group effects, especially as the biomarkers are not specific to PAD. We did not find significant correlations between the change in the levels of vWF and the change in PWD or MWD. A possible explanation for this finding is that the change in vWF levels and the change in walking distance may represent separate effects of INP treatment.

Venous blood samples may be prone to pre-analytic variation, especially in the case of non-standardized sample settings. All blood samples in our study were collected from the patients between 08.00 am and noon the day before the start of treatment, and the day after the end of treatment. All patients were told not to eat the same morning the samples were collected, and only to take their regular medications together with water. The samples were prepared and frozen according to a standardized protocol at the three study sites, and transported to the Centre for Clinical Heart Research at Oslo University Hospital for analyses at the end of the study period. To eliminate the risk for intra-assay variation, baseline and follow-up samples were analysed in batches. For all the measured biomarkers, there was a high correlation between baseline levels and levels after 12 weeks of treatment (all pairwise Spearman's rank correlation coefficients > 0.70), indicating a low pre analytic variation between the samples.

6.8 Statistical considerations

In paper I, we used pivot tables for the aggregation of data [144]. Medians for every 0.5 s of the 17 s INP cycle were normalized to the medians at time 0, and plotted, giving a pivot table for each patient at each INP level. Then, a pivot table with aggregated medians for all patients were plotted to illustrate the differences between the INP levels. Both ultrasound Doppler and laser Doppler are dynamic measurements, and signals may be disturbed by small displacements of the probes during the tests. This may result in varying data quality and outliers in the data sets. Hence, the aggregated normalized median values seemed to be the most robust measure. The differences in flow and LDF between the pressure levels could have been compared in different ways. Intuitively, one might think that the most reasonable way to calculate the differences between the pressure levels is to calculate the area under the flow and LDF curves, representing the total amount of blood flow through the INP cycle. However, we believe that the long-term

physiological effect of INP treatment is more dependent on the fluctuations in blood flow, rather than the total amount of blood flow, hence we chose to compare the intra-subject median values for maximal flow and LDF between the different pressure levels. As the data were non-normally distributed, we used Friedman test, which is a non-parametric test similar to the parametric repeated measures ANOVA, to analyse if there were differences in maximal blood flow and LDF between the INP levels [113]. Further, maximal blood flow and LDF values were compared pairwise between the different INP levels with Dunn's post-hoc tests with Bonferroni adjustment for multiple comparisons.

In paper II, we compared changes in variables from baseline to 12 weeks of treatment between the treatment group and the sham control group. Statistically, such comparisons can be done in several ways, such as comparisons of follow-up scores or comparisons of changes in scores. However, neither of these methods account for imbalance between the groups at baseline. If baseline scores are worse in the treatment group than in the control group, comparisons of follow-up scores will underestimate the treatment effect, whereas comparisons of changes in scores will overestimate the treatment effect. Therefore, we used ANCOVA, which is the recommended analysis when comparing baseline and follow-up data between groups, as it adjusts for differences between the groups at baseline [114]. ANCOVA is a regression method with the regression equation:

Follow-up score = constant + a × baseline score + b × treatment group
where a and b are estimated coefficients, and treatment group is a binary variable.

The coefficient b is the estimated effect size between the two groups, adjusted for baseline differences [114]. ANCOVA assumes that the covariate has a linear effect on the dependent variable, and that this effect is the same in both groups. The covariate should not be affected by the treatment. Further, homogeneity of variances and an approximately normal distribution of the residuals are required [145].

In paper III, we used ANCOVA to compare differences in the change of the circulating biomarker levels between the groups. In the situations where data were not normally distributed, log transformations were performed. However, this was a study of secondary outcome measures, where clinically relevant changes were difficult to assess, and no power calculations were

performed. Hence, the study may have been underpowered to detect such between-group differences. Exploratory analyses were performed by dichotomizing the differences in the baseline and post-intervention values to increased (≥ 0) or decreased (< 0), before comparing the differences in the distributions between the treatment group and the sham control group using χ^2 test. Calculations based on proportions does not provide effect estimates, however, one may argue that the effect estimates are of less importance in an exploratory setting.

In paper IV, there were no control group, but the outcome variables were measured for each subject at three different timepoints (baseline, 12 weeks, and 24 weeks). Repeated measures ANOVA can be used to assess repeated measurements of the same subjects under different conditions, or at different points over time [146]. We used repeated measures ANOVA to compare the overall change in PWD, MWD, resting ABI and post-exercise ABI over the 24-week period. Bonferroni correction was performed for post hoc comparisons of the changes between the timepoints (baseline vs. 12 weeks, and 12 weeks vs. 24 weeks).

7. ETHICAL CONSIDERATIONS

All the work related to this thesis was conducted in accordance with the Declaration of Helsinki; Ethical Principles for Medical Research Involving Human Subjects [147]. The studies were approved by the Regional Committee for Medical and Health Research Ethics in Norway (2014/1967; 2018/748) and the Department of Information Security and Privacy at Oslo University Hospital, and were registered on ClinicalTrials.gov (NCT03547817; NCT03640676). All the study subjects provided a written informed consent before they were included in the studies.

In the RCT, patients with PAD were encouraged to treat themselves at home for one hour twice daily for 12 weeks. During the treatments, the patients were sitting immobilized in a chair. Patients in the active treatment group received treatment that they possibly could benefit from, whereas patients in the sham control group received treatment that we expected not to affect their disease state. To ask patients with IC to sit immobilized for two hours per day for twelve weeks, is an eligible ethical issue to rise, especially for the patients randomized to the sham control group, despite that all participants were informed that they should continue their daily activities, and not substitute those with the INP treatment. We did not measure the patient's activity level during the intervention period, but although not significant, there was a trend towards increased

walking capacity within the sham control group after 12 weeks, which might indicate that their leg symptoms did not progress significantly due to a reduced activity level during the intervention period.

Through this project, we have tested the clinical effect of a novel treatment device. Even though the treatment principle has been used for many years, and the device has been thoroughly tested before it was approved and CE-marked as a medical device, side-effects may occur when new treatments are applied on larger patient populations. We did however not note any serious side-effects from the INP treatment in our studies.

8. FUNDING

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8.1 Industry funded research

Research collaborations between the industry and academia may raise concerns about conflicts of interests and an elevated risk of bias. A Cochrane review from 2017 concluded that sponsorship of drug and device studies by the manufacturing company leads to more favourable efficacy estimates and conclusions than sponsorship by other sources [149]. Consequently, there has been a gap between academia and the industry for many years. However, over the last decades, there are striking examples of successful collaborations between academia and the industry resulting in new important therapies for patients, such as the discovery and development of imatinib, a tyrosine kinase inhibitor used for the treatment of chronic myelogenous leukaemia [150]. The overall goal for all biomedical research is to improve health. A considerable amount of public resources are spent on research every year, and a transfer of knowledge back to the society resulting in employment and value creation is expected. Further, it is not realistic to

expect public funding of all biomedical research. Hence, enhancing ties between academia and the industry is encouraged [151]. However, any collaboration between academia and the industry must imply transparency concerning the roles of all contributors in planning, conducting, and reporting from a trial, in addition to the conflicts of interests and relevant financial disclosures. Eventually, it is up to the readers to critically evaluate strengths and weaknesses of any trial that may have impact on current knowledge.

9. CONCLUSIONS AND FUTURE PERSPECTIVES

9.1 Conclusions

- When applied to the lower leg, INP of -40 mmHg and -60 mmHg increased arterial and skin blood flow in patients with PAD. There were no significant differences in maximal arterial blood flow and skin blood flow between -60 mmHg and -40 mmHg. An INP level of -10 mmHg did not induce a significant increase in arterial blood flow and skin blood flow compared with atmospheric pressure (paper I).
- Treatment with -40 mmHg INP for one hour in the morning and one hour in the evening for 12 weeks increased PWD compared with sham treatment with -10 mmHg INP in patients with IC. For patients with baseline PWD < 200 m, treatment with -40 mmHg INP increased both PWD and MWD compared with sham treatment (paper II).
- There were no significant differences in the change in circulating levels of VCAM-1, ICAM-1, E-selectin, P-selectin, vWF, L-arginine, ADMA and SDMA after treatment with -40 mmHg INP for one hour twice daily for 12 weeks, compared with sham treatment. However, a significantly larger proportion of the patients in the treatment group had a reduction in vWF compared with the sham control group, and the concentration of vWF was significantly reduced within the treatment group after 12 weeks, which might indicate a beneficial effect of INP treatment on endothelial activation and endothelial injury (paper III).
- Patients with IC receiving treatment with -40 mmHg INP twice daily for 24 weeks increased both PWD and MWD, compared to baseline. The main increase in PWD occurred during the first 12 weeks of treatment, whereas the main increase in MWD occurred from 12 to 24 weeks of treatment (paper IV).

9.2 Future perspectives

With an aging population, the prevalence of PAD is expected to increase. The increased focus on cardiovascular risk management has dramatically improved the survival of patients with PAD, however, many patients still live with disabling leg symptoms affecting their daily life. Most patients with IC will benefit from best medical treatment and SET, and focus on availability and adherence to SET should be emphasized, as a conservative treatment strategy is medically preferable, and cost effective.

For patients with IC with limited access to SET, or who do not adhere to SET, INP treatment may serve as an adjunct to standard care. One reason for the poor adherence to SET among patients with IC, might be that the patients are exposed to pain during the exercise sessions. An improvement in PWD as observed in paper II, could be sufficient to increase the adherence to SET for some patients. For patients with IC who are considered for revascularization, but who have an elevated risk of procedure-related complications, INP treatment may also be a relevant alternative. For patients with CLI, endovascular or open surgical revascularization is the preferred treatment. Whether the effects of INP treatment in patients with IC are generalizable to patients with more sever stages of PAD is uncertain. For patients with CLI not amenable for revascularization, INP treatment could potentially be valuable, however, that should be a subject to further research.

Clinical medicine is an ever-evolving field with a continuously search for new strategies that could improve health. As health care professionals, we are committed to practice evidence-based medicine, meaning that the conscientious, explicit, and judicious use of current best evidence should support decisions about the care of individual patients [152]. The findings in this thesis may serve as a contribution to the evidence-based knowledge concerning treatment for PAD.

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SUMMARY IN NORWEGIAN

Perifer aterosklerotisk sykdom kan føre til redusert arteriell blodsirkulasjon i ekstremitetene, og rammer mer enn 235 millioner mennesker på verdensbasis. Det kliniske bildet kan være asymptomatisk, domineres av iskemiske smerter i bena ved belastning, eller manifestere seg som kritisk iskemi med hvilesmerter, iskemiske sår og utvikling av gangren. Eksponering av den affiserte ekstremiteten for pulserende undertrykk øker blodsirkulasjonen, og har blitt beskrevet som en mulig behandlingsmodalitet for pasienter med perifer aterosklerotisk sykdom siden begynnelsen av 1900-tallet. Denne behandlingsformen er nå aktualisert, da et nytt behandlingsutstyr for pulserende undertrykk er utviklet, som gjør det mulig at pasientene kan behandle seg selv hjemme. Det overordnede målet med denne doktorgraden var å undersøke de kliniske og fysiologiske effektene av pulserende undertrykksbehandling hos pasienter med perifer aterosklerotisk sykdom i underekstremitetene.

I en eksperimentell studie (artikkel I) studerte vi de akutte effektene av ulike nivåer av pulserende undertrykk på arteriell sirkulasjon og hudsirkulasjon i bena hos 16 pasienter med perifer aterosklerotisk sykdom. I en randomisert, dobbelt blindet, sham-kontrollert studie (artikkel II og III) undersøkte vi kliniske og fysiologiske effekter av pulserende undertrykksbehandling i en time, to ganger daglig i 12 uker, hos 72 pasienter med iskemiske belastningssmerter i bena. I en oppfølgingsstudie (artikkel IV) undersøkte vi kliniske effekter av behandling med pulserende undertrykk en time to ganger daglig i 24 uker hos 10 pasienter med iskemiske belastningssmerter i bena.

Pulserende undertrykk på -40 mmHg og -60 mmHg økte signifikant arteriell blodstrøm og hudblodstrøm sammenliknet med atmosfæretrykk. Pulserende undertrykk på -10 mmHg påvirket ikke arteriell blodstrøm og hudblodstrøm, og var derfor et passende trykknivå å benytte som sham-behandling i den kliniske studien. Behandling med -40 mmHg pulserende undertrykk i en time, to ganger daglig i 12 uker, økte smertefri gangdistanse sammenliknet med sham-behandling med -10 mmHg (estimert behandlingseffekt 50 m; 95% CI [11, 89]; p=0.014). For pasientene med mest uttalte symptomer (baseline smertefri gangdistanse < 200 m) var det en signifikant bedring i både smertefri og maksimal gangdistanse i behandlingsgruppen sammenliknet med sham-kontroll gruppen (hhv. estimert behandlingseffekt 42 m; 95% CI [2, 83]; p=0.042, og estimert behandlingseffekt 62 m; 95% CI [5, 118]; p=0.032). Av pasientene som ble randomisert til behandlingsgruppen hadde 25/31 (81%) en reduksjon i von Willebrand

faktor (vWF) etter 12 uker, sammenliknet med 17/30 (57%) i sham-kontroll gruppen (p=0.043). Innad i behandlingsgruppen var det en (mean [SEM]) reduksjon i konsentrasjonen av vWF på -11% [4] (p=0.019) etter 12 uker. Det ble ikke observert signifikante forskjeller i endring av ankel-arm indeks i hvile og etter belastning, hvileblodstrøm og post-iskemisk blodstrøm, livskvalitet og nivåer av sirkulerende vaskulære biomarkører mellom gruppene etter 12 ukers behandling. Etter behandling med pulserende undertrykk med -40 mmHg i 24 uker, var det en signifikant økning i både smertefri og maksimal gangdistanse sammenliknet med baseline (hhv. p=0.006 og p=0.012). Økningen i smertefri gangdistanse kom hovedsakelig i løpet av de første 12 ukene, mens økningen i maksimal gangdistanse hovedsakelig kom fra 12-24 ukers behandling.

Pulserende undertrykk på -40 mmHg gav en akutt økning i arteriell blodstrøm og hudblodstrøm i foten hos pasienter med perifer aterosklerotisk sykdom. Behandling med pulserende undertrykk på -40 mmHg en time to ganger daglig i 12 uker økte gangdistansen sammenliknet med sham-behandling hos pasienter med iskemiske belastningssmerter i bena. En signifikant andel av pasientene i behandlingsgruppen fikk en reduksjon i vWF, og nivåene av vWF ble redusert i behandlingsgruppen etter 12 uker, hvilket kan tyde på en positiv effekt på arteriell endotelaktivering og endotelskade. Den positive effekten på gangdistanse så ut til å vedvare fra 12 til 24 ukers behandling.

PAPERS

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ORIGINAL RESEARCH

The acute effects of different levels of intermittent negative pressure on peripheral circulation in patients with peripheral artery disease

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Arterial blood flow, intermittent negative pressure, peripheral artery disease, skin blood flow.

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Abstract

Intermittent negative pressure (INP) applied to the lower leg induces acute increase in arterial and skin blood flow. The aim of this study was to identify the optimal level of INP to increase blood flow in patients with lower extremity peripheral artery disease (PAD). We investigated the acute effects of different levels of INP in 16 subjects (7 women and 9 men, mean (SD) age 71(8) years) diagnosed with PAD. During application of INP in a pressure chamber sealed below the knee, arterial blood flow was continuously recorded in the dorsalis pedis artery or tibialis posterior artery (ultrasound Doppler), and skin blood flow was continuously recorded at the pulp of the first toe (laser Doppler). Different pressure levels (0, −10, −20, −40, and −60 mmHg) were tested in randomized order. Maximal arterial blood flow relative to baseline (median [25th, 75th percentiles]) was: 0 mmHg; 1.08 (1.02, 1.13), −10 mmHg; 1.11 (1.07, 1.17), −20 mmHg; 1.18 (1.11, 1.32), −40 mmHg; 1.39 (1.27, 1.91) and −60 mmHg; 1.48 (1.37, 1.78). Maximal laser Doppler flux (LDF) relative to baseline was: 0 mmHg; 1.06 (1.02, 1.12), −10 mmHg; 1.08 (1.05, 1.16) −20 mmHg; 1.12 (1.06, 1.27), −40 mmHg; 1.24 (1.14, 1.50) and −60 mmHg; 1.35 (1.10, 1.70). There were significantly higher maximal arterial blood flow and maximal LDF at −40 mmHg compared with −10 mmHg ($P = 0.001$ and $P = 0.025$, respectively). There were no significant differences in maximal arterial blood flow and maximal LDF between 0 and −10 mmHg (both $P = 1.0$), or between −40 and −60 mmHg (both $P = 1.0$). INP of −40 mmHg was the lowest negative pressure level that increased blood flow.

Introduction

Peripheral artery disease (PAD) comprise all conditions resulting in obstruction of blood flow in arteries, exclusive of the coronary, and intracranial vessels (Ouriel 2001). Atherosclerosis leading to obstruction of blood flow to the lower extremities is a common manifestation of PAD. Lower extremity PAD may lead to ischemia causing pain while walking that is relieved by rest, termed intermittent claudication. This may progress to critical limb ischemia resulting in pain at rest, tissue loss, and gangrene (Norgren et al. 2007).

Standard treatment for PAD includes smoking cessation, pharmacological therapy with antiplatelet agents and

statins, and supervised physical exercise (Conte et al. 2015). Supervised exercise programs are recommended to improve functional status and reduce leg symptoms and should be discussed as a treatment option for patients with intermittent claudication before revascularization (Gerhard-Herman et al. 2017). However, a systematic review from 2016 reported that only 1 in 3 patients with intermittent claudication were suitable for and willing to participate in supervised exercise programs (Harwood et al. 2016). For patients with severe PAD, endovascular or open surgery may be options to achieve revascularization, but the ability to perform endovascular or open surgery depends on the localization and extent of the disease. A significant proportion of the patients also have severe co-morbidities (Ouriel 2001), which may contraindicate surgery.

The effects of intermittent negative pressure (INP) to improve blood flow in patients with PAD have been investigated since the early 20th century (Sinkowitz and Gottlieb 1917; Landis and Gibbon 1933; Herrmann and Reid 1934; Herrmann 1935). A number of studies have described different devices applying INP by alternately removing air from and venting a pressure chamber sealed around the patients' leg or lower body (Sinkowitz and Gottlieb 1917; Landis and Gibbon 1933; Herrmann and Reid 1934; Smyth 1969; Himmelstrup et al. 1991; Sundby et al. 2017). In 1969, Smyth et al. applied intermittent negative pressure to the lower limbs of patients with different stages of PAD and observed improvement in resting and post ischemic blood flow and walking distance after 6 weeks of treatment (Smyth, 1969). A Danish study from 1983 showed that locally applied constant negative pressure increased vascular resistance and reduced subcutaneous blood flow, but the vasoconstriction was abolished by local nervous blockade induced by low doses of lidocaine injected subcutaneously, suggesting that the vasoconstriction was due to a local sympathetic veno-arterial axon reflex mechanism, which constricts the arterioles when veins are distended (Skagen and Henriksen 1983). One study has demonstrated that INP combined with heated water applied to the arm was effective to prevent hypothermia in patients undergoing laparotomy (Rein et al. 2007). In this study, it was suggested that the observed increase in blood flow was due to increased pressure difference between the arterial and venous system, and by avoidance of the veno-arterial reflex. A recent study on healthy volunteers from our research group showed that 2 min of constant negative pressure applied to the lower extremities decreased blood velocity in the dorsalis pedis artery (ADP) or tibialis posterior artery (ATP), and skin blood flow (Sundby et al. 2016). The same study demonstrated that INP of −40 mmHg applied to the lower extremities increased maximal arterial blood velocity 44% (95% CI 33–55) above baseline. In another study using the same experimental setup on patients with PAD, maximal arterial blood velocity increased 46% (95% CI 36–57) above baseline (Sundby et al. 2017). This effect thus seems comparable between healthy volunteers and patients with PAD. The three latter studies reported INP levels of −40 mmHg, in cycles of 10 sec negative pressure, and 7 sec atmospheric pressure to be effective to increase arterial and skin blood flow (Rein et al. 2007; Sundby et al. 2016; 2017).

Although several studies have demonstrated increased blood flow in the extremity during application of INP, the optimal level of INP to improve blood flow is unknown. Our hypothesis was that blood flow in the foot increases with increasing magnitude of INP until a certain level. Hence, the aim of the present study was to identify the optimal level of INP applied in sequences of 10 sec negative pressure and 7 sec atmospheric pressure to increase blood flow in the lower extremities in subjects with PAD.

Methods

Participants

Study subjects were recruited from the out-patient clinic at the Department of Vascular Surgery, Oslo University Hospital, Oslo, Norway. Subjects with resting ankle-brachial index (ABI) <0.9 and symptomatic claudication or radiological detected PAD were included. Subjects undergoing recent (less than three months) endovascular or open surgical revascularization were considered not eligible for the study.

Experimental setup and measurements

We registered age, sex, weight, height, comorbidities, smoking status, medications, main localization of the disease, previous revascularization, and patient reported maximal walking distance for all subjects based on a questionnaire and the subjects' medical record at Oslo University Hospital.

All subjects were encouraged not to eat, and to refrain from tobacco and caffeine two hours before the experiments. Measurements were conducted in a temperature stable environment of 22–24°C. The subjects´ most symptomatic leg was chosen as the test leg.

ABI was measured after 5 min of rest, with the subject in supine position using a continuous wave 8 MHz Doppler probe (Macrolab, STR Teknikk, Aalesund, Norway), in accordance with the guidelines from the American Heart Association (Aboyans et al. 2012).

Pulse volume recording (PVR) amplitude was measured with an air-plethysmography cuff (Macrolab, STR

Teknikk, Aalesund, Norway) placed at the lower leg above the malleoli. Normal arterial inflow to the extremity is pulsatile, leading to measurable changes in lower limb volume within each cardiac cycle (Hashimoto et al. 2016), and in the case of PAD, the waveform of PVR becomes dampened.

We measured the peak systolic velocity and the diameter of the ADP or ATP using a triplex ultrasound scanner (GE LOGIQ 9 Ultrasound, Wauwatosa, Wisconsin, USA). A pulsed 10 MHz Doppler probe (SD-50, GE Vingmed Ultrasound, Horten, Norway) was used to measure the arterial blood velocity during application of INP. The probe was fixed to the foot with surgical tape above the ATP or ADP, at the place where the best Doppler signal was achieved. Based on the peak systolic velocity measured with the ultrasound triplex scan, the Doppler ultrasound was calibrated to record the exact arterial blood velocity in the ATP or ADP.

A laser Doppler flow meter was used to monitor microvascular blood perfusion in acral skin using a laser Doppler probe attached to the pulp of the first toe (Peri-Flux 5000, Perimed AB, Jarfalla, Sweden). The same probe recorded skin temperature.

Pressure inside the pressure chamber was recorded by a digital manometer (Macrolab, STR Teknikk, Aalesund, Norway).

Systemic blood pressure was measured non-invasively beat-by-beat, by a Finometer (FMS, Finapres medical systems BV, Arnhem, Netherlands) attached to the third finger of the right arm.

With the subject sitting in a chair, all probes were connected, and the foot was carefully introduced into the pressure chamber. The foot arch was placed on a positioner to avoid the front foot and the heel to touch the pressure chamber. The pressure chamber was sealed just below the knee using a customized thermoplastic elastomer seal and coupled by air hoses to a control unit (FlowOx, Otivio AS, Oslo, Norway) that generated INP by actively removing air from and passively venting the pressure chamber (Fig. 1). The control unit was programmed to apply time sequences of 10 sec of negative pressure and 7 sec of atmospheric pressure during all tests. Before the start of the experiments, adequate signals were confirmed. The subjects were encouraged to sit relaxed with approximately 130° flexion in the knee joint during the experiment. For all the subjects, experimental data were first sampled in a 5-min sequence at atmospheric pressure, before INP sequences, each lasting 5 min were sampled. Pressure levels of −10, −20, −40 and −60 mmHg were tested with a 5-min wash-out period between the tests. To account for possible carry-over effects between the pressure levels, the test order of the INP levels was randomized using an online randomization software (Research Randomizer, www.randomizer.org).

Figure 1. Intermittent negative pressure generated in a pressure chamber sealed around the lower leg, by control unit that is actively removing air from and passively venting the pressure chamber.

Data recorded during the tests were sampled at 300 Hz, and averaged beat-by-beat, gated by the R-waves of a three lead ECG, using custom-made software (REGIST3, Morten Eriksen, University of Oslo, Norway). The software calculated beat-by-beat blood flow in the ADP or ATP during the tests by adding information on the angle of insonation and the vessel diameter measured with the ultrasound triplex scan before application of INP. The beat-by-beat data were resampled to 2 Hz for further analyses. Obviously erroneous data due to for example motion artefacts were removed from the dataset, giving 15 complete INP cycles, each lasting 17 sec, for each subject at each pressure level.

Statistical analyses

Descriptive statistics are presented as mean (standard deviation [SD]) or median (25th, 75th percentile). Shapiro–Wilks tests were performed to assess if the flow and LDF data were normally distributed at every 0.5 sec of the INP cycles, giving $P < 0.001$ for all tests. Therefore, these data were not assumed to have a normal distribution.

For each subject and INP level, median arterial blood flow, laser Doppler flux (LDF), and mean arterial pressure (MAP) every 0.5 sec of the 17 sec cycle of INP were divided by the median value at time 0 (baseline) for the pressure level being tested, giving an intra-subject relative median value for each 0.5 sec of the 17 sec INP cycle. The aggregated medians of flow and LDF for all subjects

were plotted to illustrate the differences between the pressure levels. From the intra-subject median values, the maximal values were found for each INP level, giving the maximal blood flow and LDF relative to baseline for that subject and INP level. We used Friedman test for non-normally distributed data to examine the overall null hypothesis of no significant differences in any of the rank sums of maximal blood flow, LDF and mean arterial pressure (MAP) between the INP levels (Eisinga et al. 2017). For pairwise comparisons of maximal blood flow and LDF between the INP levels, Dunn's post hoc tests were performed with Bonferroni adjustment for multiple comparisons. We used SPSS (IBM Statistics for Windows, Version 25.0. IBM Corp., Armonk, NY, USA) and SigmaPlot (SigmaPlot, Version 12.0, Systat Software Inc., San Jose, CA, USA) for statistical analyses and plotting of data.

A recent study on patients with PAD reported a mean (SD) blood velocity in ADP of 6.7 (3.3) cm/sec (Sundby et al. 2017). To detect an increase in blood velocity of 40% during INP, at least 12 subjects must be included in the current study given a significance level of 0.05 and a power of 80%.

Ethics

The project was approved by the Regional Committee for Medical and Health Research Ethics in Norway (ref: 2014/1967) and registered at ClinicalTrials.gov (ref: SD0321063). Written informed consent was obtained from all subjects before the start of the experiments.

Results

Sixteen subjects with PAD Fontaine stage I (1 subject) and Fontaine stage II (15 subjects) were included in the study (Table 1). Patient reported maximal walking distance was 225 (100, 500) meters and ABI was 0.62 (0.15). Twelve subjects had femoropopliteal disease, two had aortoiliac disease, and two had infrapopliteal disease. Six subjects had previously undergone revascularization of the tested leg.

Maximal blood flow during the 17 sec cycles at pressure levels of −60 and −40 mmHg were reached after 3 sec and 2 sec, respectively, followed by a gradual decrease in flow until 10 sec, when the negative pressure was turned off. During the 7 sec with atmospheric pressure, the blood flow decreased below baseline, before returning to baseline after 17 sec (Fig. 2).

Maximal LDF during the 17 sec cycles at pressure levels of −60 and −40 mmHg were reached after 3 sec, followed by a gradual decrease in LDF until the negative pressure was turned off after 10 sec. During the last 7 sec with atmospheric pressure, we observed a slight increase in LDF before returning to baseline after 17 sec (Fig. 3).

Table 1. Subject's characteristics, n = 16.

Age, years*	71 (8)
Male sex	9 (56)
Body mass index, kg/m^2*	23.5 (3.1)
Test leg right	11 (69)
Symptomatic bilateral PAD	9 (56)
Ankle-brachial index test leg*	0.62 (0.15)
Pulse volume recording test leg, mm*	8.3 (3.4)
Blood flow measured in dorsalis pedis artery	12 (75)
Smoking status, current smoker/ex-smoker/nonsmoker	3 (19)/8 (50)/5 (31)
Diabetes mellitus	1 (6)
Chronic renal failure	1 (6)
Hypercholesterolemia	9 (56)
Hypertension	10 (63)
Coronary artery disease	8 (50)
Cerebrovascular disease	2 (13)
Antiplatelet agents	14 (88)
Lipid lowering agents	15 (94)
Antihypertensive agents	11 (69)
Patient reported maximal walking distance, meters†	225 (100, 500)
Fontaine stage	
I	1 (6)
IIa	9 (56)
IIb	6 (38)
III	0 (0)
IV	0 (0)
Main localization of disease	
Aortoilliac	2 (13)
Feomorpoliteal	12 (75)
Infrapopliteal	2 (13)
Previous revascularization of test leg	
Endovascular	5 (31)
Open surgery	1 (6)

Values are number (%) unless otherwise stated.
*Mean (standard deviation).
†Median (25th, 75th percentile).

Maximal arterial blood flow for each pressure level, relative to baseline was: 0 mmHg; 1.08 (1.02, 1.13), −10 mmHg; 1.11 (1.07, 1.17), −20 mmHg; 1.18 (1.11, 1.32), −40 mmHg; 1.39 (1.27, 1.91) and −60 mmHg; 1.48 (1.37, 1.78). Maximal LDF for each pressure level relative to baseline was: 0 mmHg; 1.06 (1.02, 1.12), −10 mmHg; 1.08 (1.05, 1.16), −20 mmHg; 1.12 (1.06, 1.27), −40 mmHg; 1.24 (1.14, 1.50) and −60 mmHg; 1.35 (1.10, 1.70) (Fig. 4).

Overall, there were significant differences in maximal arterial blood flow and maximal LDF between the pressure levels (both $P < 0.001$). For pairwise comparisons of maximal arterial blood flow, there were significant differences between: 0 and −40 mmHg ($P < 0.001$), 0 and

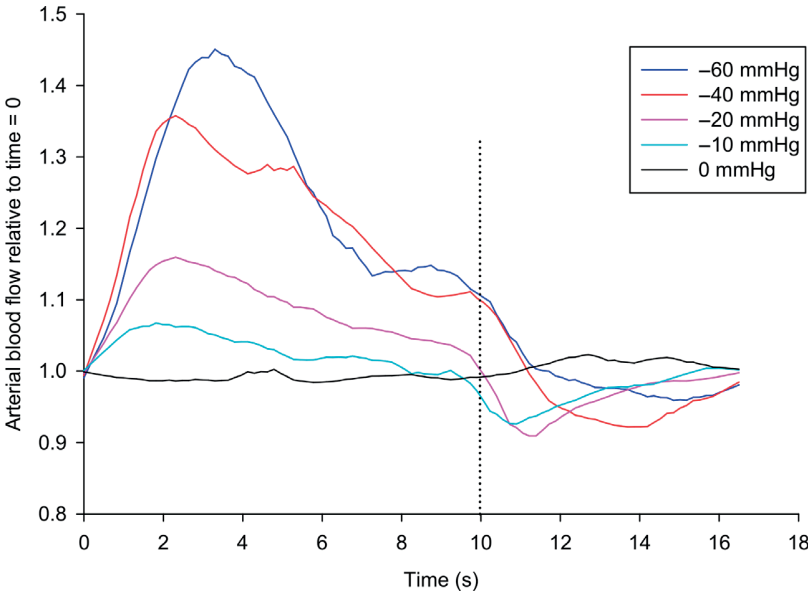

Figure 2. Arterial blood flow in the foot during the 17 sec cycles of intermittent negative pressure. Aggregated medians relative to baseline (time = 0 sec) plotted every 0.5 sec for all patients (n = 16) at each pressure level. Dashed line indicates switch from negative pressure to atmospheric pressure.

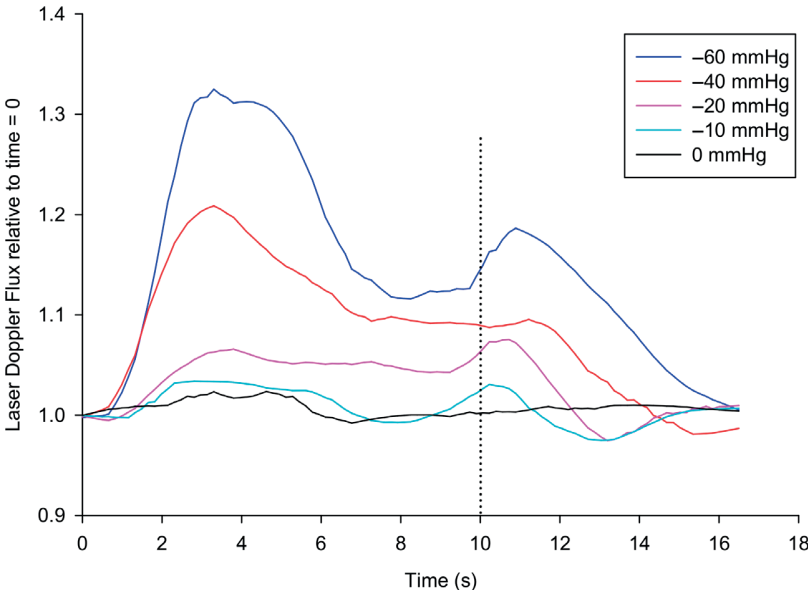

Figure 3. Laser Doppler Flux in acral skin of the foot during the 17 sec cycles of intermittent negative pressure. Aggregated medians relative to baseline (time = 0 sec) plotted every 0.5 sec for all patients (n = 16) at each pressure level. Dashed line indicates switch from negative pressure to atmospheric pressure.

−60 mmHg ($P < 0.001$), −10 and −40 mmHg ($P = 0.001$), −10 and −60 mmHg ($P < 0.001$) and −20 and −60 mmHg ($P = 0.005$). For pairwise comparisons of maximal LDF, there were significant differences between: 0 and −40 mmHg ($P = 0.001$), 0 and −60 mmHg ($P < 0.001$), −10 and −40 mmHg

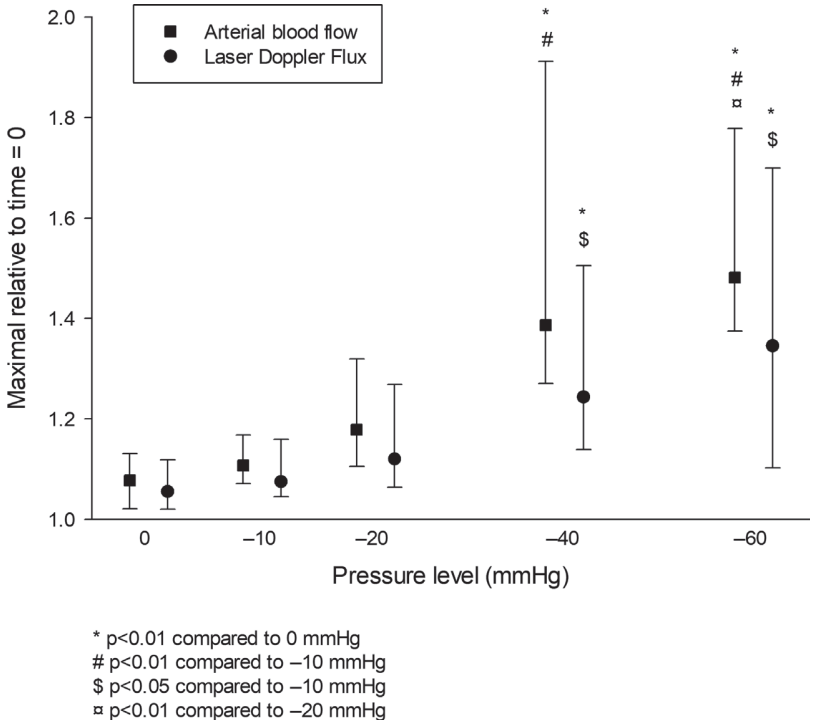

Figure 4. Maximal arterial blood flow and Laser Doppler Flux during the 17 sec cycles of intermittent negative pressure. Medians and 25th–75th percentiles for all patients (n = 16) relative to baseline (time = 0 sec) at different pressure levels.

(P = 0.025) and -10 mmHg and -60 mmHg (P = 0.012). There were no significant differences in maximal arterial blood flow or maximal LDF between 0 and −10 mmHg (both P = 1.0) or between −40 and −60 mmHg (both P = 1.0). There was no significant difference in the maximal change of MAP between the pressure levels (P = 0.434).

Discussion

The main finding of the present study was that application of INP to the lower extremity with pressure levels of −40 mmHg and −60 mmHg increased maximal arterial blood flow and skin blood flow in the foot compared to 0 and −10 mmHg in subjects with PAD. There were no significant differences in maximal arterial blood flow and skin blood flow between −60 and −40 mmHg. Hence, −40 mmHg was the lowest level of negative pressure that induced such changes. An INP level of -10 mmHg did not significantly induce an acute increase in maximal arterial blood flow and skin blood flow compared with atmospheric pressure alone.

The finding that an INP level of −40 mmHg induced acute increase in arterial blood flow and skin blood flow is in line with previous studies (Sundby et al. 2016; Sundby et al. 2017; 2018). However, the mechanisms of action leading to increased blood flow during INP are not well described in the literature. According to Poiseuille's law for laminar flow through a cylindric tube, the flow is dependent on the pressure difference between the two sides of the tube, the radius of the tube, and the viscosity of the liquid flowing through the tube (Pfitzner 1976). INP may affect both the pressure differences between the arterial and venous side of the capillary bed, as well as the vessel diameter. One previous study observed an increased blood flow also in a 5-min period after INP treatment was ended (Sundby et al. 2017). This suggests that the leg benefits from the treatment for a longer time than just during the period of INP. This may be explained by the increased blood flow during INP leading to increased shear stress between the blood and the endothelium of the arterial wall, and thereby inducing flow-mediated vasodilatation (Joannides et al. 1995). Hence, flow-mediated vasodilatation may be one physiological explanation for the potential clinical benefits of INP treatment for patients with PAD that have been presented in a number of studies (Herrmann and Reid, (1934); Sundby et al. 2017; Himmelstrup et al. 1991; Smyth 1969; Mehlsen et al. 1993).

The theoretical rationale of applying INP instead of constant negative pressure is to avoid the veno-arterial reflex mechanism that induces vasoconstriction on the

arterial side when veins become distended (Skagen and Henriksen 1983). Activation of this reflex is probably dependent both on the level of negative pressure applied and the length of the negative pressure periods and atmospheric pressure periods. If the negative pressure periods are shortened or the atmospheric pressure periods are lengthened, there is a possibility that a higher pressure difference (higher INP level) will induce an even greater increase in blood flow. Previous studies of INP treatment of patients with PAD have tested pressure levels of -120, −150, and −200 mmHg (Landis and Gibbon 1933; Smyth 1969; Gill and Walder 1974; Himmelstrup et al. 1991; Mehlsen et al. 1993), but the way in which negative pressure was applied, and the length of negative pressure periods and atmospheric pressure periods varies between the different studies.

Sundby et al. assessed pain during application of INP in patients with PAD, finding a mean verbal numerical rating pain scale of 0 at an INP level of −40 mmHg (Sundby et al. 2017). Even though the potential side effects of INP treatment are few and the discomfort for the patients is little, it is reasonable to not expose patients to local pressure changes that are higher than what is necessary to achieve optimal blood flow.

The level of evidence of the clinical effects of INP treatment in the literature is scarce and should be a subject for further research. In this study, we demonstrated that a pressure level of −10 mmHg did not significantly increase arterial blood flow and skin blood flow. This might thus be the INP level of a sham device in a randomized sham-controlled trial designed to explore if INP can contribute in the treatment of patients with lower extremity PAD.

There are some limitations in this study. It was not possible to measure the vessel diameters in the pressure chamber during INP. Therefore, the diameters of ADP and ATP were measured before application of INP, and arterial blood velocity was measured during application of INP. Hence, the flow calculations were based upon the vessel diameter before application of INP. If application of INP leads to a shear stress induced vasodilatation, this may have led to underestimation of arterial blood flow. We used a pulsed 10 MHz ultrasound Doppler probe fixed to the subjects' feet over the ADP or ATP to monitor blood flow during the application of INP. Small displacements of the probe caused by the subject changing the position of the leg during the experiment may decrease the quality of the Doppler signal, leading to periods of underestimation of blood flow. Furthermore, this study was conducted in a standardized environment on subjects with PAD, and one should be careful with generalization of the results to other patient groups or settings.

This study concludes that INP treatment of subjects with PAD with pressure levels of −40 and −60 mmHg applied in cycles of 10 sec of negative pressure and 7 sec of atmospheric pressure induced acute increase in arterial and skin blood flow. INP of −40 mmHg was the lowest negative pressure level that increased blood flow.

Conflict of Interest

HH is employed by Otivio with funding from the Norwegian Research Council (NFR grant no: 285758). IM is the CSO, co-founder and a shareholder in Otivio AS. Otivio AS has the commercial rights to the INP technology (FlowOx) used in the study. None of the other authors have any conflicts of interest, financial, or otherwise. The authors alone are responsible for the content and writing of the paper.

Data Availability Statement

All data supporting the results in this paper will be publicly available on the repository Figshare.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.:

Table S1. Pairwise comparisons of the different levels of intermittent negative pressure.

II

A randomized controlled trial of treatment with intermittent negative pressure for intermittent claudication

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ABSTRACT

Objective: We investigated the effects of lower extremity intermittent negative pressure (INP) treatment for 1 hour two times daily for 12 weeks on the walking distance of patients with intermittent claudication (IC).

Methods: Patients with IC were randomized to treatment with −40 mm Hg INP (treatment group) or −10 mm Hg INP (sham control group). Pain-free walking distance (PWD) and maximal walking distance (MWD) on a treadmill, resting and postexercise ankle-brachial index, resting and postischemic blood flow (plethysmography), and quality of life (EQ-5D-5L and Vascuqol-6) were measured at baseline and after 12 weeks of treatment.

Results: A total of 72 patients were randomized, and 63 had data available for the intention-to-treat analyses. The between-group comparisons showed a significant change in the PWD, favoring the treatment group over the sham control group (estimated treatment effect, 50 m; 95% confidence interval [CI], 11-89; $P = .014$). The PWD had increased by 68 m ($P < .001$) in the treatment group and 18 m ($P = .064$) in the sham control group. No significant difference was found in the change in the MWD between the two groups (estimated treatment effect, 42 m; 95% CI, −14 to 97; $P = .139$). The MWD had increased by 62 m ($P = .006$) in the treatment group and 20 m ($P = .265$) in the sham control group. For patients with a baseline PWD of <200 m (n = 56), significant changes had occurred in both PWD and MWD between the two groups, favoring the treatment group (estimated treatment effect, 42 m; 95% CI, 2-83; $P = .042$; and estimated treatment effect, 62 m; 95% CI, 5-118; $P = .032$; respectively). Both overall and for the group of patients with a PWD <200 m, no significant differences were found in the changes in the resting and postexercise ankle-brachial index, resting and postischemic blood flow, or quality of life parameters between the two groups.

Conclusions: Treatment with −40 mm Hg INP increased the PWD compared with sham treatment in patients with IC. For the patients with a baseline PWD of <200 m, an increase was found in both PWD and MWD compared with sham treatment. (J Vasc Surg 2021;73:1750-8.)

Keywords: Intermittent claudication; Intermittent negative pressure treatment; Peripheral artery disease

Peripheral artery disease (PAD) affects >235 million people globally, and the prevalence is increasing.[1] Intermittent claudication (IC) is a common symptom in patients with PAD characterized by muscle discomfort in the lower limb that is provoked by exercise and relieved by rest[2] and is associated with reduced ambulatory activity and quality of life.[3,4] Participation in supervised exercise therapy (SET) programs increase the walking capacity of patients with IC[5,6] and is the first-line treatment, together with smoking cessation and pharmacologic secondary prevention.[7] However, the availability of SET programs is low,[8] and many patients are unwilling or unable to participate.[9] Consequently, home-based exercise programs and different treatment devices have been suggested as alternative treatment options.[10-12]

Methods using intermittent negative pressure (INP) applied to the lower body or extremities to improve

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Author conflict of interest: H.H. is employed by Otivio AS with funding from The Research Council of Norway (grant 285758). I.M. is the chief strategy officer and shareholder in Otivio AS. Otivio AS has the commercial rights to the intermittent negative pressure technology used in the present study. E.M.P., L.Ø.H., A.S., and J.H. have no conflicts of interests. Otivio AS was not involved in the study design; collection, analysis, or interpretation of data; manuscript writing; or the decision to submit the manuscript for publication.

Additional material for this article may be found online at www.jvascsurg.org.

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blood flow in patients with PAD have been described since the early 20th century.[13-15] Several studies have suggested positive effects on walking distance in patients with IC.[16-18] However, two recent studies did not find any additional effects of INP treatment on walking capacity.[19,20] Because the treatment intensity and duration varies among previous studies and the results differ, the clinical effect of INP treatment for patients with IC remains uncertain.

The aim of the present study was to investigate the clinical effects of treatment with −40 mm Hg INP for 1 hour in the morning and 1 hour in the evening for 12 weeks in patients with IC. We hypothesized that this treatment would improve the pain-free walking distance (PWD) and maximal walking distance (MWD) compared with sham treatment.

ARTICLE HIGHLIGHTS

- **Type of Research:** A multicenter, prospective, randomized controlled trial
- **Key Findings:** Treatment of intermittent claudication with lower extremity intermittent negative pressure for 1 hour twice daily for 12 weeks increased the pain-free walking distance in the treatment group (n = 38) receiving −40 mm Hg intermittent negative pressure compared with the sham control group (n = 34) receiving −10 mm Hg intermittent negative pressure.
- **Take Home Message:** Treatment with lower extremity intermittent negative pressure increased the pain-free walking distance compared with sham treatment for patients with intermittent claudication. For the patients with the most symptomatic disease, an increase occurred in both pain-free and maximal walking distance compared with sham treatment.

METHODS

Participants. We performed a multicenter trial, enrolling patients from the outpatient clinics at three vascular surgery departments in Norway (Oslo University Hospital, Oslo; Sørlandet Hospital, Kristiansand; and St Olavs Hospital, Trondheim) from January to September 2019. Data collection was completed in December 2019. Patients with an ankle-brachial index (ABI) of ≤0.9 or incompressible arteries and a radiologic diagnosis of PAD and IC were assessed for eligibility. Patients who had been scheduled for or who had undergone endovascular or open surgical revascularization within the previous 3 months were considered ineligible. The exclusion criteria were: inability to provide an informed consent; inability to perform a treadmill test; inability to independently operate the treatment device; the presence of severe heart disease; the presence of severe chronic obstructive pulmonary disease; and a baseline MWD of >1000 m measured on a treadmill with a ramp protocol.[21] All patients were offered the best medical treatment according to the guidelines from the European Society of Cardiology and the European Society for Vascular Surgery.[7]

INP treatment. INP was applied in a pressure chamber sealed around the lower leg. A pump unit (FlowOx 2.0; Otivio AS, Oslo, Norway) removed air from, and vented, the pressure chamber (Fig 1), producing alternating 10 seconds negative pressure and 7 seconds atmospheric pressure. The patients in the treatment group received −40 mm Hg INP, and the sham control group received −10 mm Hg INP. The devices were otherwise identical. These INP levels were chosen according to the findings from a recent study from our research group demonstrating that −40 mm Hg INP induced an acute increase in blood flow in the treated extremity in patients with PAD, in contrast to −10 mm Hg INP which did not significantly affect blood flow.[22]

All patients were instructed to treat themselves at home for 1 hour in the morning and 1 hour in the evening for 12 weeks. They were trained in the use of the INP device before the start of treatment. The daily treatment time was recorded by the device, allowing for the analysis of compliance data after the intervention period. To avoid the direct effects of treatment on the test results, the patients were instructed not to use the device on the day of the 12-week follow-up examination.

Randomization and blinding. Patients were randomized to the treatment group or sham control group in a 1:1 ratio using a computer-generated randomization list. Labeling of the treatment devices was performed by the producer (Otivio AS) by a person not involved in patient recruitment or data collection. The patients and personnel with patient contact during the study period were unaware of the group allocation. The statistical analyses were also performed without knowledge of the treatment group.

Clinical evaluation and measurements. Clinical evaluation and measurements were performed by the same person at baseline and after 12 weeks of treatment. The primary outcome measures were the changes in PWD and MWD. The pain-free walking time and maximal walking time were measured with the patients walking on a treadmill using a ramp protocol at a constant speed of 3.2 km/h starting at a 0% slope and increasing the slope by 2% every 2 minutes.[21] The patients were asked to specify the most limiting leg after the baseline treadmill test, which was chosen as the treatment leg.

Before the treadmill test, the resting ABI was measured with the patient in a supine position according to the guidelines from the American Heart Association.[23] The

Fig 1. Device for lower extremity intermittent negative pressure (INP) treatment. INP is generated in a pressure chamber sealed around the patient's lower leg by a pump unit that removes air from, and vents, the pressure chamber. Provided by Otivio AS, Oslo, Norway.

postexercise ABI was measured with the patient in supine position within 1 minute after the end of the treadmill test.

Resting blood flow was measured with the patient in the supine position using strain-gauge plethysmography (Domed Filtrass Angio, Krailling, Germany) of the lower leg at the point of maximal circumference. The plethysmograph records the rate of change in volume expansion for the area under the strain-gauge during proximal venous occlusion on the thigh, and the blood flow values are calculated.[24] Postischemic blood flow was measured using the same strain-gauge plethysmograph after 3 minutes of arterial occlusion obtained by inflating the thigh cuff to 250 mm Hg. For safety reasons, patients who had previously undergone bypass surgery in the leg were excluded from the present examination, as were patients who experienced unbearable pain during occlusion.

All the patients were requested to complete the EQ-5D-5L and Vascuqol-6 quality of life questionnaires at baseline and after 12 weeks of treatment. The EQ-5D-5L questionnaire consists of a visual analog scale and a descriptive system.[25] Using each patient's score in the descriptive system, an index value was calculated (EQ-5D-5L index) based on a value set validated for Denmark.[26] The Vascuqol-6 is a health-related quality of life questionnaire for patients with PAD validated for Norway,[27] consisting of six disease-specific items with a total score ranging from 6 to 24, with a higher score indicating better health.

Statistical analysis. The data are presented as the mean ± standard deviation for continuous variables and numbers and percentages for categorical variables, unless otherwise stated. Normality was assessed by histograms, Q-Q plots, and residual plots. The baseline characteristics between the groups were compared using independent samples *t*-tests for continuous variables and χ^2 tests for categorical variables. Differences within the groups were analyzed using paired sampled *t*-tests. Differences between groups were evaluated using univariate analysis of covariance, adjusting for differences in baseline data.[28] All subjects with pre- and post-treatment data were included in the intention-to-treat analyses. *P* values ≤.05 were considered statistically significant. Analyses were performed using Stata, version 16 (StataCorp, College Station, Tex).

In a comparable population of patients with IC, the pain-free walking time on a treadmill was 146 ± 112 seconds.[29] Assuming an increase in pain-free walking time of 87 seconds (76 m) as a clinically important difference,[30] 26 patients per treatment arm were required to detect a treatment effect, given 80% power and a 5% significance level. Assuming a withdrawal rate of 25%, we aimed to include 35 patients in each group.

Ethics. The Regional Committee for Medical and Health Research Ethics in Norway approved the present study (reference no. 2018/748), which was registered at ClinicalTrials.gov (identifier, NCT03640676). All the patients provided written informed consent before inclusion.

RESULTS

A total of 85 patients were assessed for eligibility. Of the 85 patients, 2 did not meet the inclusion criteria (baseline MWD >1000 m), and 11 patients declined to participate, leaving 72 patients (85%) for randomization (Oslo University Hospital, n = 46; St Olavs Hospital, n = 5; Sørlandet Hospital, n = 21; Fig 2). At baseline, a significantly higher prevalence of diabetes was present in the treatment group compared with the sham control group (*P* = .008). No significant differences were found between the two groups for all other demographic variables (Table I).

Walking distance. The between-group comparisons showed a significant difference in the change in the PWD, favoring the treatment group compared with the sham control group (estimated treatment effect, 50 m; 95% confidence interval [CI], 11-89; *P* = .014; Table II). At baseline, the PWD was 109 ± 70 m in the treatment group and 111 ± 77 m in the sham control group (Table I). After 12 weeks of treatment, an increase in the PWD of 68 m had occurred in the treatment group (95% CI, 33-103; *P* < .001) and 18 m in the sham control group (95% CI, −1 to 38; *P* = .064; Fig 3).

The between-group comparisons showed no significant differences in the change in the MWD after 12 weeks of INP treatment (estimated treatment effect, 42 m; 95% CI, −14 to 97; *P* = .14; Table II). The baseline MWD was 267 ± 177 m in the treatment group and 263 ± 170 m in the sham control group (Table I). After 12 weeks of

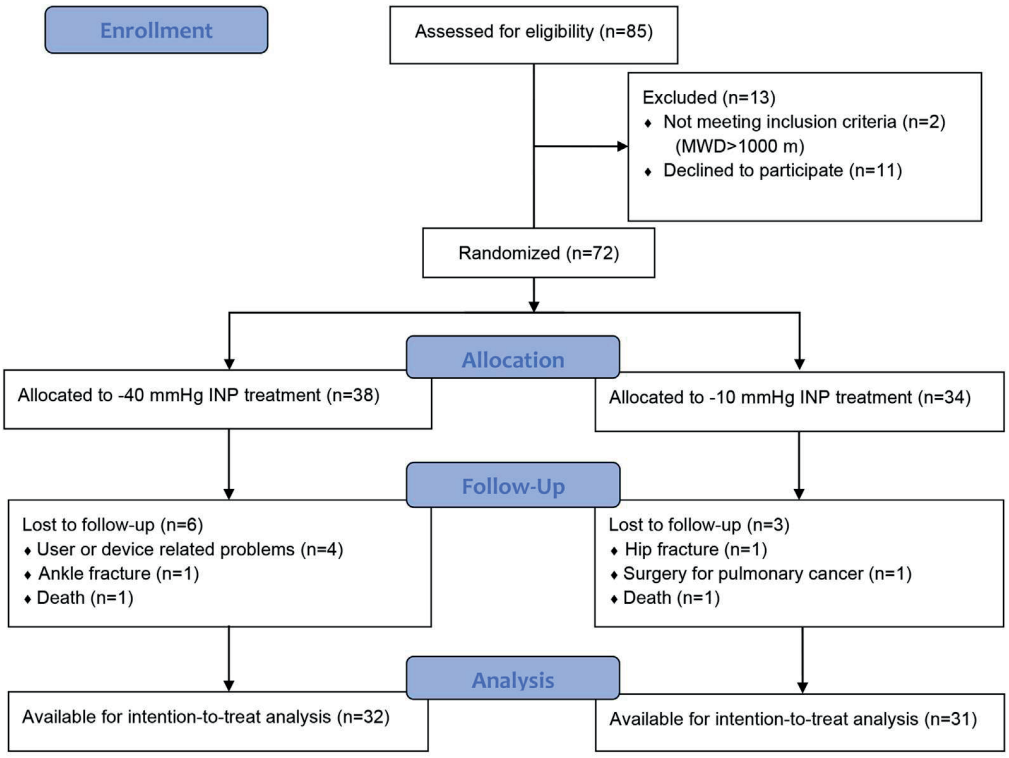

Fig 2. CONSORT (consolidated standards of reporting trials) flow diagram showing inclusion, exclusion, treatment allocation, and outcomes. *INP,* Intermittent negative pressure; *MWD,* maximal walking distance.

treatment, an increase had occurred in the MWD of 62 m in the treatment group (95% CI, 19-105; $P = .006$) and 20 m in the sham control group (95% CI, −16 to 57; $P = .27$; Fig 3).

Of the 63 patients who had completed the 12-week intervention period, 56 (89%) had had a baseline PWD of <200 m. For these patients, the between-group comparisons showed statistically significant differences in the changes in both PWD and MWD, favoring the treatment group (estimated treatment effect, 42 m; 95% CI, 2-83; $P = .042$; and estimated treatment effect, 62 m; 95% CI, 5-118; $P = .032$, respectively; Table III).

ABI, blood flow measurements, and quality of life. At baseline, the resting ABI was 0.53 ± 0.16 in the treatment group and 0.56 ± 0.15 in the sham control group (Table I). No significant changes were found in the resting or postexercise ABIs across the groups ($P = .65$ and $P = .19$, respectively), and the plethysmography measurements did not show significant changes in the resting or post-ischemic blood flow across the groups ($P = .34$ and $P = .58$, respectively) after 12 weeks of treatment. Furthermore, no significant changes were observed in the quality of life questionnaire scores (EQ-5D-5L index, $P = .67$; EQ-5D-5L visual analog scale score, $P = .29$; Vascuqol-6, $P = .89$) across the groups after 12 weeks of treatment (Table II). Relative within-group changes for all outcome variables after 12 weeks of treatment are illustrated in the Supplementary Fig (online only).

Compliance, discontinuation, and adverse events. The mean daily treatment time was 1.8 ± 0.2 hours in the treatment group and 1.8 ± 0.5 hours in the sham control group ($P = .63$). Six patients in the treatment group and three patients in the sham control group were lost to follow-up (Fig 2). Four patients in the treatment group discontinued treatment because of issues related to use of the treatment device. One patient in the treatment group and one patient in the sham control group discontinued because of severe trauma (ankle fracture and hip fracture, respectively), and one patient in the sham control group discontinued because of surgery for pulmonary cancer. One patient in each group died, both of cardiac arrest (unrelated to the INP sessions) during the 12-week intervention period. No further serious adverse events were reported.

DISCUSSION

The main finding from the present study was that treatment with −40 mm Hg INP for 1 hour in the morning and 1 hour in the evening for 12 weeks increased the PWD compared with sham treatment for patients with IC. For the patients with a baseline PWD of <200 m

Table I. Baseline patient characteristics

Variable	Treatment group (n = 38)	Sham control group (n = 34)	*P* value
Age, years	72 ± 8	73 ± 6	.59[a]
Male sex	25 (66)	26 (76)	.32[b]
Body mass index, kg/m^2	27.3 ± 4.2	26.9 ± 4.0	.74[a]
Smoking			.92[b]
Current	14 (37)	11 (32)	
Previous	19 (50)	18 (53)	
Never	15 (39)	5 (15)	
Comorbidity			
Diabetes mellitus	18 (47)	6 (18)	.008[b]
Chronic renal failure	5 (13)	4 (12)	.86[b]
Hypertension	32 (84)	28 (82)	.83[b]
Hypercholesterolemia	22 (58)	27 (79)	.051[b]
Coronary artery disease	17 (45)	18 (53)	.49[b]
Cerebrovascular disease	8 (21)	8 (24)	.80[b]
Antiplatelet agent	32 (84)	27 (79)	.60[b]
Anticoagulant agent	6 (16)	8 (24)	.41[b]
Statin	32 (84)	31 (91)	.37[b]
Antihypertensive agent	34 (89)	31 (91)	.81[b]
Treated leg, right	19 (50)	22 (65)	.21[b]
Disease location			.81[b]
Suprainguinal	6 (16)	4 (12)	
Infrainguinal	23 (61)	23 (68)	
Supra- and infrainguinal	9 (24)	7 (21)	
Previous revascularization in treated leg	16 (42)	12 (35)	.55[b]
PWD, m	109 ± 70	111 ± 77	.90[a]
MWD, m	267 ± 177	263 ± 170	.94[a]
Resting ABI	0.53 ± 0.16	0.56 ± 0.15	.33[a]
Postexercise ABI	0.35 ± 0.17	0.33 ± 0.13	.52[a]
Vascuqol-6	13.4 ± 3.0	13.7 ± 3.3	.68[a]
EQ-5D-5L index	0.65 ± 0.20	0.70 ± 0.12	.20[a]

ABI, Ankle-brachial index; *MWD*, maximal walking distance; *PWD*, pain-free walking distance.
Data presented as mean ± standard deviation for continuous variables and as number (%) for categorical variables.
[a]Independent samples *t*-test.
[b]χ^2 Test.

(clinically classified as Fontaine IIb, corresponding to Rutherford class 2-3), both PWD and MWD increased in the treatment group compared with the sham control group.

To the best of our knowledge, the present study is one of the first double-blind randomized controlled trials to show that INP treatment increases the walking distance for patients with IC. Our findings are in line with the results reported by a placebo controlled study from Denmark, which also described an effect on walking distances.[18] However, only the within-group changes had been reported in that study.[18] Older studies have reported similar findings for patients with PAD,[13,16,17,31] but these were mainly case reports and patient series. However, two recent placebo controlled trials concluded that INP treatment does not provide additional effects to SET or home-based physical activity and lifestyle changes in increasing the walking capacity in patients with IC.[19,20] One explanation might be that the effect of increased physical activity surpasses the potential effects of INP treatment. Another explanation might be that the patients were treated with INP for only 30 and 40 minutes two and three times each week for 6 weeks, respectively, because that INP system required in-hospital treatment instead of at-home treatment. Hence, the frequency and length of the INP treatments were significantly lower than those used in the present study. Because INP applied to the lower leg induces acute

Table II. Analysis of covariance for all patients (N = 63)

Variable	No. available for analysis	Estimated treatment effect (95% CI)	*P* value
PWD, m	63	50 (11-89)	.014
MWD, m	63	42 (−14 to 97)	.14
Resting ABI	60	0.01 (−0.04 to 0.07)	.65
Postexercise ABI	47	0.04 (−0.02 to 0.10)	.19
Blood flow, mL/100 mL tissue			
Resting	59	0.5 (−0.6 to 1.6)	.34
Postischemic	53	0.4 (−1.1 to 2.0)	.58
Vascuqol-6	63	−0.11 (−1.53 to 1.31)	.89
EQ-5D-5L index	57	0.01 (−0.05 to 0.08)	.67
EQ-5D-5L VAS	63	5.3 (−4.7 to 15.4)	.29

ABI, Ankle-brachial index; *CI,* confidence interval; *MWD,* maximal walking distance; *PWD,* pain-free walking distance; *VAS,* visual analog scale.

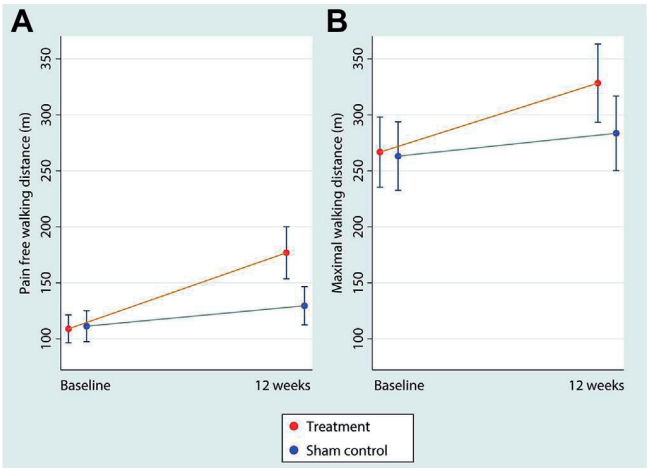

Fig 3. Pain-free walking distance (PWD) **(A)** and maximal walking distance (MWD) **(B)** at baseline and after 12 weeks of treatment. *Dots* indicate mean values; *error bars,* standard errors.

rhythmical fluctuations in blood flow,[22,32,33] which might promote long-term favorable effects leading to an increased walking capacity, it is reasonable to assume that a higher treatment frequency would be favorable and might also be necessary to achieve clinical effects.

In patients with a baseline PWD of <200 m, we observed an increase in both PWD and MWD in the treatment group compared with the sham control group. Although determined from a subgroup analysis, this finding indicates that the patients with the most symptomatic disease might benefit the most from INP treatment. Multiple studies have documented the beneficial effects of SET programs on walking capacity, functional status, and quality of life in patients with IC.[5,34-38] However, a systematic review from 2016 concluded that only one third of patients with IC were suitable for or willing to undertake SET.[9] Hence, the current guidelines recommending SET might not be applicable to most patients with IC. Although SET should be the first choice of treatment for patients with IC, INP treatment might be a useful supplement when SET is unavailable or for patients unable or unwilling to participate in SET.

The between-group changes in PWD in the present study were lower than assumed in the power calculations. However, to the best of our knowledge, no consensus has been reached regarding the minimal clinically important difference in walking performance after interventions in patients with IC. It is probably dependent on the disease severity, comorbidities, and the patient's subjective judgment. Pharmacologic agents such as cilostazol and pentoxifylline have market approval in Europe and the United States, with an indication of improving leg symptoms in patients with IC. A Cochrane review from 2014 estimated that cilostazol could increase the PWD by 31 m (95% CI, 22-40) and MWD by 43 m (95% CI, 18-68) compared with placebo.[39] Another Cochrane review from 2015 reported an improvement in PWD of −33.8% to 73.9% and in MWD of 1.2% to 156% with pentoxifylline. However, statistical tests were not performed because of insufficient data.[40] In the present study, we found an estimated treatment effect on the PWD of 50 m and a treatment effect for patients with a PWD <200 m of 42 m for PWD and 62 m for MWD. Thus, INP treatment is competitive to drug treatment in increasing the walking capacity of patients with IC.

Treatment of the calf or foot using intermittent pneumatic compression (IPC) has also been suggested to improve the walking distance for patients with IC.[11] Both INP and IPC increase arterial blood flow acutely when applied to the lower limb,[22,33,41] which might increase arterial shear stress, thereby inducing flow-mediated vasodilatation.[22,42] However, IPC is applied over a smaller area on the calf or foot and might not have the same microvascular effects on the whole lower leg compared with INP.

Endovascular or open surgery can be considered for patients with IC who have severely disabling symptoms and do not respond to SET.[7,43,44] A Cochrane review from

Table III. Analysis of covariance for baseline pain-free walking distance <200 m subgroup (N = 56)

Variable	No. available for analysis	Estimated treatment effect (95% CI)	*P* value
PWD, m	56	42 (2-83)	.042
MWD, m	56	62 (5-118)	.032
Resting ABI	53	0.00 (−0.06 to 0.06)	.91
Postexercise ABI	44	0.04 (−0.03 to 0.10)	.27
Blood flow, mL/100 mL tissue			
Resting	53	0.7 (−0.4 to 1.8)	.20
Postischemic	47	0.3 (−1.4 to 2.0)	.74
Vascuqol-6	56	−0.25 (−1.80 to 1.29)	.75
EQ-5D-5L index	53	0.02 (−0.05 to 0.08)	.61
EQ-5D-5L VAS	55	4.5 (−6.4 to 15.5)	.41

ABI, Ankle-brachial index; *CI*, confidence interval; *MWD*, maximal walking distance; *PWD*, pain-free walking distance; *VAS*, visual analog scale.

2018, which compared endovascular revascularization and conservative treatment of IC, reported a moderate effect on MWD and a large effect on PWD after 6 to 12 months.[45] However, no clear differences were shown between the groups after long-term follow-up.[45] Different outcome measures and study designs did not allow for direct comparisons to the findings in the present study. However, the effect of INP treatment after 3 months should be subject to further research.

In the present study, we did not observe any differences across the groups in the resting or postexercise ABI after 12 weeks of treatment. This is in line with the findings from a systematic review on the effects of exercise on IC, reporting an increased walking capacity without finding significant changes in the ABI.[5] Although we found an effect of INP treatment on walking distance, we did not find any differences in the quality of life parameters across the groups after 12 weeks of treatment. This does not correspond with the findings from studies investigating the effects of SET in IC.[36,38] One explanation might be that the improvement in the quality of life after participation in SET is also related to other factors than just the improvement in walking distance, such as increased physical activity and social interactions, which might not be obtained using INP treatment alone. Another explanation might be that the present study was underpowered to detect changes in quality of life across the groups.

The treatment group received −40 mm Hg INP, which is the standard INP level provided by the Conformitè Europëenne–marked FlowOx system commercially available in Europe. This INP level seemed to be well tolerated, and it is possible that a higher level could have been used. However, in a previous study from our research group, we did not observe any significant difference in the acute increase in arterial or skin blood flow at −60 mm Hg INP compared with −40 mm Hg INP.[22] Whether subgroups of patients could benefit from a higher INP level requires further investigation.

The prevalence of diabetes was higher in the treatment group than in the sham control group. Patients with diabetes are more prone to microangiopathy. Hence, the clinical effects observed in the present study could have resulted from positive effects on the arterial circulation or microcirculation, or both.

One patient in each group died of cardiac arrest during the intervention period. No clinical evidence was found to support a causal relationship between these events and the use of the treatment device or participation in the present study. The number of deaths in the present study did not allow for further statistical interpretations but underscores the high mortality for patients with symptomatic PAD.[46]

The present study had some limitations. We used −10 mm Hg INP in the sham device to make it appear identical to the active device without affecting the arterial blood flow. The similarity in compliance between the groups and the low withdrawal rate in the sham control group indicates that the patients really were unaware of their treatment allocation. However, a small effect might have resulted from the repetitive exposure to −10 mm Hg INP, leading to an underestimation of the treatment effect. More patients were lost to follow-up in the treatment group than in the sham control group because of user- or device-related problems. The use of the device requires some technical, cognitive, and motor capacity. In addition, some patients with a very small circumference of the lower leg might experience difficulty in achieving airtightness of the pressure chamber. Hence, the difference in those lost to follow-up between the two groups might have been random. Measurements of PWD is based on a subjective report of the onset of pain by the patient during the treadmill test. Thus, the results might have been affected by the fact that individuals might perform differently when they are being observed. However, in a double-blind, randomized controlled trial, the risk of bias from this phenomenon seems low. The patients were recruited from

the vascular surgery departments at three hospitals in Norway. Thus, one should be careful about generalizing the results to other patient populations or settings. However, whether the results from the present study are also applicable to patients with more severe stages of PAD should be the subject of further research.

CONCLUSIONS

The results from the present study have shown that treatment with −40 mm Hg INP for 1 hour in the morning and 1 hour in the evening for 12 weeks increased the PWD compared with sham treatment in patients with IC. For patients with a baseline PWD of <200 m, treatment with −40 mm Hg INP increased both PWD and MWD compared with sham treatment.

AUTHOR CONTRIBUTIONS

Conception and design: HH, IM, JH
Analysis and interpretation: HH, EP, LH, JH
Data collection: HH, EP, JH
Writing the article: HH
Critical revision of the article: HH, EP, LH, IM, AS, JH
Final approval of the article: HH, EP, LH, IM, AS, JH
Statistical analysis: HH, LH
Obtained funding: Not applicable
Overall responsibility: HH

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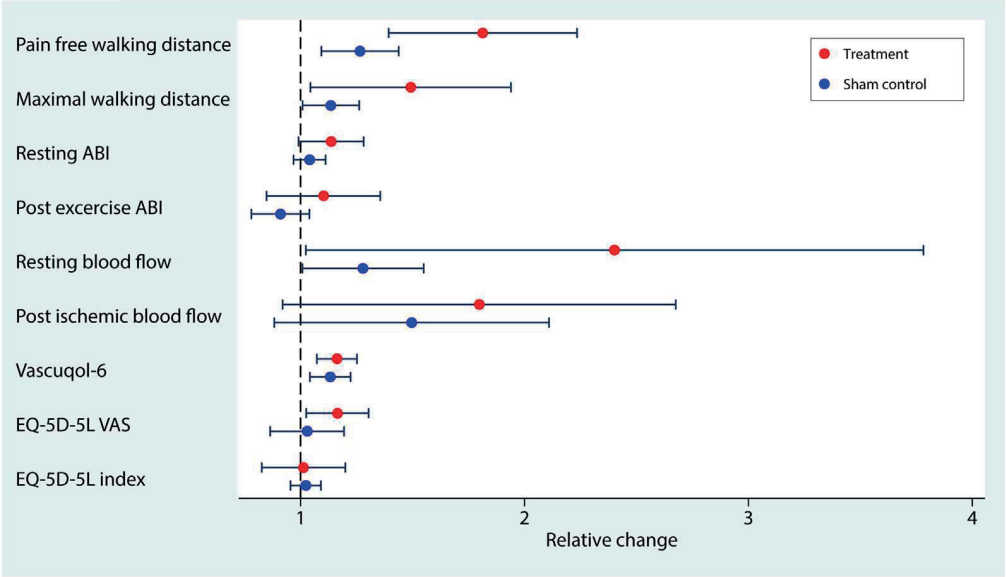

Supplementary Fig (online only). Relative within-group changes for all outcome variables. *Dots* indicate mean values; *error bars*, 95% confidence intervals. *ABI*, Ankle-brachial index.

III

Original Article

VASCULAR MEDICINE

Vascular Medicine
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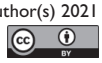

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Effects of intermittent negative pressure treatment on circulating vascular biomarkers in patients with intermittent claudication

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Abstract

The aim of this study was to investigate the effects of lower extremity intermittent negative pressure (INP) treatment for 1 hour twice daily for 12 weeks, on circulating vascular biomarkers in patients with intermittent claudication. Patients were randomized to treatment with –40 mmHg INP (treatment group), or –10 mmHg INP (sham control group). Venous blood samples were collected at baseline and after 12 weeks, and concentrations of vascular adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), E-selectin, P-selectin, von Willebrand factor (vWF), L-arginine, asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) were analyzed. A larger proportion of the patients in the treatment group (25/31) had a reduction in vWF levels after 12 weeks, compared to the sham control group (17/30) ($p = 0.043$). Within the treatment group there was a significant mean (SEM) reduction in the concentration of vWF of –11% (4) ($p = 0.019$), whereas there was no significant change in the levels of vWF in the sham control group (1% (6); $p = 0.85$). There were no significant differences in the change of any of the biomarker levels between the groups after 12 weeks of treatment. In conclusion, there were no differences in the change of the circulating levels of the measured biomarkers between the treatment group and the sham control group after 12 weeks of INP treatment. However, the observed changes in vWF might indicate a beneficial effect of INP treatment on endothelial activation and endothelial injury. **Clinicaltrials.gov Identifier:** NCT03640676

Keywords

intermittent claudication, intermittent negative pressure treatment, peripheral artery disease (PAD), vascular endothelium, vascular medicine

Introduction

Atherosclerosis is a multifocal disease causing build-up of atheromatous lesions in the arterial wall that may impede blood flow.[1] In peripheral artery disease (PAD), atherosclerotic stenosis or occlusion of the arteries to the lower extremities may result in ischemic muscle pain in the legs provoked by exercise that is relieved by rest, a clinical sign known as intermittent claudication (IC).[2]

Atherosclerotic activity is associated with altered levels of circulating biochemical substances indicative of vascular inflammation, endothelial damage, endothelial dysfunction, or atheromatous plaque instability.[3–5] In the early phase of the atherosclerotic process, the endothelium becomes activated by an atherogenic or proinflammatory stimuli, leading to upregulation and expression of adhesion molecules, recruiting monocytes and T lymphocytes to the arterial wall.[6] Chemoattractant cytokines stimulate monocytes and T lymphocytes to enter the arterial intima,[7] and monocytes derive into macrophages expressing receptors for internalization and oxidation of lipoproteins. The lipid loaded macrophages replicate inside the intima and secrete proinflammatory cytokines and reactive oxygen species

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that amplify the inflammatory response, causing progression of the atheromatous lesion.

Patients with PAD have increased risk of cardiovascular morbidity and mortality, and the aims of the treatment are twofold: first, reduction of cardiovascular risk factors; second, treatment of the leg symptoms. Standard treatment for patients diagnosed with IC is pharmacological secondary prevention with antiplatelet agents and cholesterol lowering agents, smoking cessation, and participation in supervised exercise therapy (SET) programs.[8] A systematic review from 2014 concluded that physical activity positively affected key biomarkers in atherosclerosis,[9] and a study from 2011 concluded that 8 weeks of SET increased walking distance, and reduced plasma levels of the specific endothelium-derived inflammatory markers E-selectin and intracellular adhesion molecule-1 in patients with PAD.[10] However, the adherence and availability to SET programs are low,[11] and other treatment options have been proposed. Repetitive exposure of the symptomatic leg to alternating pressure differences has been suggested to increase walking distance and improve wound healing in patients with PAD in a number of studies;[12–19] however, as two studies did not show any additional effect on walking capacity in patients with IC, the treatment effect has been debated.[20,21] Recently, a randomized, double blind sham-controlled trial from our research group showed that lower extremity intermittent negative pressure (INP) treatment for 1 hour twice daily for 12 weeks increased the pain-free walking distance in patients with IC.[22] However, the physiological and biochemical mechanisms explaining the clinical improvements in patients with IC after INP treatment are not fully understood. To explore this further, we aimed to investigate the potential effect of lower extremity INP treatment for 1 hour twice daily for 12 weeks on circulating levels of vascular adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), E-selectin, P-selectin, von Willebrand factor (vWF), L-arginine, asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) as markers of vascular inflammation, endothelial injury, and endothelial function.

Methods

Participants, intermittent negative pressure (INP) treatment, randomization, and blinding

This was an exploratory study of secondary outcome measures from a randomized controlled multicenter trial.[22] Patients were enrolled from the outpatient clinics at three vascular surgery departments in Norway (Oslo University Hospital, Oslo; Sørlandet Hospital, Kristiansand; and St Olavs Hospital, Trondheim) between January and September 2019. Data collection was completed in December 2019. Patients with an ankle–brachial index (ABI) $\leqslant 0.9$, or incompressible leg arteries and radiologically diagnosed PAD, and IC were assessed for eligibility. Exclusion criteria were: endovascular or open surgical revascularization within the last 3 months, inability to perform a treadmill test, inability to independently operate the INP-treatment device, baseline maximal walking distance > 1000 m, and severe chronic obstructive pulmonary disease or severe heart disease corresponding to New York Heart Association Functional Class IV.[23] Eligible patients were randomized to treatment with –40 mmHg INP (treatment group) or –10 mmHg INP (sham control group) in a 1:1 ratio using a computer-generated randomization list. The levels of INP used in the treatment device and in the sham device, and their impact on blood flow, has been documented in a previous study.[24] Patients and personnel with patient contact during the study period were blinded to the group allocation, as were the laboratory technologists performing the laboratory analyses. Treatment with INP was applied in a pressure chamber sealed around the lower leg by a pump unit (FlowOx 2.0; Otivio AS, Oslo, Norway) that removed air from and vented the pressure chamber in sequences of 10 seconds negative pressure and 7 seconds atmospheric pressure (Figure 1). Pain-free and maximal walking distance were measured with a treadmill test[25] at baseline and after 12 weeks of treatment. The patients were instructed to treat the most limiting leg at the baseline test for 1 hour in the morning and 1 hour in the evening for 12 weeks.

Figure 1. Intermittent negative pressure generated in a pressure chamber sealed around the patient's lower leg by a pump unit that is removing air from and venting the pressure chamber.
Source: Otivio AS/Bastian Fjeld.

Laboratory methods

Venous blood samples were collected from all patients between 08:00 and 12:00 the day before the start of the intervention period, and the day after the intervention period. Patients were instructed not to eat the same morning the samples were collected but were advised to take their regular medication with water. Serum was prepared within 1 hour by centrifugation in room temperature at 2500 $\times$ g for 15 minutes. EDTA and citrated blood were collected and stored on ice until platelet-poor plasma was obtained,

and centrifugated within 30 minutes at 2800 × g for 20 minutes. All samples were frozen at –80°C. Serum was used for analysis of VCAM-1, ICAM-1, and E-selectin, citrated plasma was used for analysis of P-selectin and vWF, and EDTA-plasma was used for analysis of L-arginine, ADMA, and SDMA. Commercial ELISA kits were used for VCAM-1, ICAM-1, E-selectin, P-selectin (R&D Systems Europe, Abingdon, UK), and vWF (Asserachrom vWF Ag, Stago Diagnostica, Asnieres, France). Intra-assay coefficients of variations (CVs) were 3.3%, 2.1%, 6.5%, 3.9%, and 9.5%, respectively. L-arginine, ADMA, and SDMA were determined by high performance liquid chromatography (HPLC) and precolumn derivatization with *o*-phthaldialdehyde (OPA) (Sigma Chemicals Co., St Louis, MO, USA). CVs were 5.9%, 7.0%, and 9.6%, respectively. All samples were analyzed in batches to eliminate intra-assay variability. Routine blood samples (hemoglobin, thrombocytes, leukocytes, creatinine, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, glycosylated hemoglobin, C-reactive protein, and albumin) were analyzed with conventional methods.

Statistics

Data are presented as median (25th, 75th percentile) or mean (SEM) for continuous variables, and number (%) for categorical variables. Concentrations of VCAM-1, ICAM-1, E-selectin, P-selectin, vWF, L-arginine, ADMA, and SDMA at baseline were plotted against concentrations after 12 weeks of treatment. The differences in baseline and post-intervention values were dichotomized to increased ($\geqslant 0$) or decreased (< 0), and differences in the distributions between the treatment group and the sham control group were compared using χ^2 test. Normality was assessed with histograms, Q–Q plots, and residual plots. In the situations where data were not normally distributed, log transformations were performed. Differences in the changes of the biomarker levels between the groups were compared using analysis of covariance (ANCOVA). Differences within the groups were compared using paired sampled *t*-test. All subjects with pre- and post-data available were included in the analyses. Spearman correlation coefficients were calculated to evaluate the correlation between the change in the measured biomarkers, and the change in pain-free and maximal walking distance after 12 weeks. A $p < 0.05$ was considered statistically significant. Analyses were performed using Stata, Release 16 (StataCorp LLC, College Station, TX, USA).

As this was an exploratory study of secondary outcome measures, and clinically significant changes were difficult to estimate, a separate sample size calculation for the present study was not performed.

Ethics

The study was approved by the Regional Committee for Medical and Health Research Ethics in Norway (ref: 2018/748) and registered on ClinicalTrials.gov (NCT03640676). Written informed consent was obtained from all patients before inclusion.

Results

In total, 85 patients were assessed for eligibility and 72 patients were randomized. A CONSORT flow diagram has recently been published.[22] Demographic variables are presented in Table 1. Sixty-three patients completed the 12-week intervention period and were available for analyses. Serum and EDTA samples were available for all patients, whereas citrated plasma samples were available for 61; thus, vWF and P-selectin analyses were lacking for two patients. For all the measured biomarkers, there was a high correlation between baseline levels and levels after 12 weeks of treatment (all pairwise Spearman's rank correlation coefficients [r_s] > 0.70). Of the patients randomized to the treatment group, 25/31 (81%) had a reduction in vWF levels after 12 weeks, compared to 17/30 (57%) in the sham control group ($p = 0.043$) (Figure 2).

There were no statistically significant differences in the change of any of the biomarker levels between the groups after 12 weeks of treatment as determined by ANCOVA (Table 2). At baseline, the mean (SEM) concentration of vWF was 200% (11) in the treatment group and 189% (9) in the sham control group. Within the treatment group there was a significant reduction in the concentration of vWF of –11% (4) ($p = 0.019$), whereas there was no significant change in the levels of vWF in the sham control group (1% (6); $p = 0.85$). The changes in vWF within the groups are illustrated in Figure 3. For all the other measured biomarkers, no significant within-group changes were shown. There was no significant correlation between the change in vWF and the change in pain-free walking distance ($r_s = -0.22$, $p = 0.088$), and no significant correlation between the change in vWF and the change in maximal walking distance ($r_s = -0.07$, $p = 0.61$) after 12 weeks.

Discussion

The main finding of the present study was that a significantly larger proportion of the patients receiving treatment with –40 mmHg INP twice daily for 12 weeks had a reduction in vWF, compared to the patients receiving sham treatment. Further, we observed a significant reduction in the plasma concentration of vWF within the treatment group after 12 weeks; however, no differences between the groups were observed. For VCAM-1, ICAM-1, E-selectin, P-selectin, L-arginine, ADMA, and SDMA no significant changes were observed after 12 weeks of INP treatment.

In a recent paper from our research group, we concluded that INP treatment increased pain-free walking distance compared to sham treatment in patients with IC,[22] a finding that is in line with several previous studies.[13–19] However, to our knowledge, the present study is the first to explore the effects of INP treatment on a molecular level. vWF is a glycoprotein synthesized and stored in endothelial cells and plays important roles in primary hemostasis by mediating

Table 1. Baseline characteristics of patients.

Variable	Treatment ($n = 38$)	Sham control ($n = 34$)
Age, years	72 (68, 75)	73 (69, 78)
Male sex	25 (66)	26 (76)
Body mass index, kg/m^2	26.4 (24.7, 29.9)	26.7 (23.7, 29.6)
Smoking		
Current	14 (37)	11 (32)
Previous	19 (50)	18 (53)
Never	15(39)	5 (15)
Diabetes mellitus	18 (47)	6 (18)
Chronic renal failure	5 (13)	4 (12)
Hypertension	32 (84)	28 (82)
Hypercholesterolemia	22 (58)	27 (79)
Coronary artery disease	17 (45)	18 (53)
Cerebrovascular disease	8 (21)	8 (24)
Antiplatelet agent	32 (84)	27 (79)
Anticoagulant agent	6 (16)	8 (24)
Statin	32 (84)	31 (91)
Antihypertensive agent	34 (89)	31 (91)
Resting ankle–brachial index	0.50 (0.43, 0.67)	0.57 (0.46, 0.64)
Pain-free walking distance (m)	87 (45, 140)	86 (50, 151)
Maximal walking distance (m)	242 (149, 375)	236 (106, 375)
Hemoglobin (g/dL)	14.3 (13.1, 15.0)	14.6 (13.4, 15.5)
Thrombocytes ($\times 10^9$/L)	256 (191, 285)	238 (183, 276)
Leucocytes ($\times 10^9$/L)	8.0 (6.7, 9.8)	8.1 (6.1, 8.9)
Creatinine (μmol/L)	89 (75, 115)	83 (71, 103)
eGFR (mL/min/1.73 m^2)	67 (51, 78)	72 (61, 89)
HbA1c (mmol/mol)	44 (39, 60)	38 (36, 43)
Cholesterol (mmol/L)	3.9 (3.5, 4.4)	4.1 (3.6, 4.7)
High-density lipoprotein (mmol/L)	1.1 (1.0, 1.3)	1.3 (1.1, 1.7)
Low-density lipoprotein (mmol/L)	2.4 (1.9, 2.9)	2.3 (1.9, 2.8)
Triglycerides (mmol/L)	1.3 (1.0, 2.1)	1.3 (0.8, 1.8)
C-reactive protein (mg/L)	2 (1, 4)	2 (1, 3)
Albumin (g/L)	45 (43, 47)	44 (42, 46)

Continuous variables are presented as median (25th, 75th percentiles). Categorical variables are presented as number (%).
eGFR, estimated glomerular filtration rate; HbA1c, glycosylated hemoglobin.

platelet adhesion and aggregation to sites of endothelial injury, and also mediates coagulation by stabilizing coagulation factor VIII in the circulation.[26] Circulating levels of vWF are increased in patients with PAD[27] and are suggested to have a prognostic value for patency after infrainguinal bypass grafting, and for future risk of cardiovascular events.[28,29] Although based on proportion calculations and within-group comparisons, the observed reduction in vWF after INP treatment suggests that INP treatment could reduce prothrombotic endothelial properties in patients with PAD. Exposure of the limb to INP acutely increases fluctuations in arterial and skin blood flow,[24] leading to increased arterial shear stress followed by flow-mediated dilation.[30] Both flow-mediated dilation and circulating levels of vWF are markers of endothelial function, and an inverse relationship between the levels of circulating vWF and the flow-mediated dilation response has been suggested.[31] Hence, a reduction in circulating levels of vWF after INP treatment may indicate a positive effect on endothelial function and endothelial injury. There were no significant correlations between the change in the levels of vWF and the change in pain-free walking distance or maximal walking distance. A possible explanation for this finding is that the change in vWF and the change in walking distance probably represent separate effects of INP treatment.

Nitric oxide (NO) is a potent vasodilator that plays an important role in vascular homeostasis through antiatherogenic and antiproliferative effects on the arterial wall. The release of NO in response to arterial shear stress promotes flow-mediated dilation.[32] NO is produced in the endothelial cells by the enzymatic conversion of L-arginine mediated by nitric oxide synthase (NOS). ADMA and SDMA are endogenous products of proteolysis, which inhibit NO synthesis. ADMA inhibits NOS by competing with L-arginine on the active site of NOS, while SDMA inhibits the cellular uptake of the NO precursor homoarginine. ADMA and SDMA are sensitive markers for endothelial dysfunction, and homoarginine/ADMA ratio and homoarginine/SDMA ratio are suggested to be independent predictors for long-term cardiovascular mortality and events in patients with lower extremity PAD.[33] In the present study, we did not

Table 2. Changes in levels of circulating vascular biomarkers from baseline to 12 weeks ($n = 63$ patients).

Variable	Baseline	12 weeks	Change from baseline to 12 weeks	*p*-value within groups[a]	*p*-value between groups[b]
VCAM-1 (ng/mL)					0.53
Treatment	908 (29)	900 (28)	–8 (13)	0.58	
Sham control	879 (49)	884 (48)	4 (8)	0.61	
Log ICAM-1 (ng/mL)					0.77
Treatment	5.667 (0.043)	5.627 (0.037)	–0.039 (0.029)	0.18	
Sham control	5.553 (0.034)	5.543 (0.030)	–0.010 (0.022)	0.66	
Log E-selectin (ng/mL)					0.36
Treatment	3.739 (0.084)	3.707 (0.083)	–0.031 (0.020)	0.13	
Sham control	3.575 (0.069)	3.576 (0.070)	0.001 (0.019)	0.95	
Log P-selectin (ng/mL)					0.46
Treatment	3.721 (0.051)	3.687 (0.048)	–0.034 (0.018)	0.071	
Sham control	3.746 (0.052)	3.729 (0.047)	–0.017 (0.024)	0.47	
von Willebrand factor (%)					0.15
Treatment	200 (11)	189 (11)	–11 (4)	0.019	
Sham control	189 (9)	190 (9)	1 (6)	0.85	
L-arginine (μmol/mL)					0.56
Treatment	38 (2)	38 (1)	0 (1)	0.81	
Sham control	40 (1)	39 (1)	–2 (1)	0.13	
ADMA (μmol/mL)					0.71
Treatment	0.43 (0.01)	0.44 (0.01)	0.01 (0.01)	0.33	
Sham control	0.43 (0.01)	0.44 (0.01)	0.01 (0.01)	0.20	
Log SDMA (μmol/mL)					0.27
Treatment	–1.008 (0.046)	–0.977 (0.050)	0.031 (0.022)	0.16	
Sham control	–1.055 (0.065)	–1.054 (0.065)	0.001 (0.018)	0.95	
L-arginine/SDMA ratio					0.50
Treatment	91 (5)	89 (4)	–2 (3)	0.46	
Sham control	96 (4)	91 (4)	–6 (3)	0.06	

Data presented as mean (SEM). Log natural logarithm.
[a]Paired sample *t*-test.
[b]Analysis of covariance.
ADMA, asymmetric dimethylarginine; ICAM-1, intracellular adhesion molecule-1; SDMA, symmetric dimethylarginine; VCAM-1, vascular adhesion molecule-1.

observe any change in L-arginine, ADMA or SDMA after 12 weeks of INP treatment. This may indicate that despite improving walking capacity in patients with IC,[22] INP treatment does not seem to affect the NO synthesis pathway measured at a systemic level.

Atherosclerosis is a chronic inflammatory process that has predilection to discrete regions in the arterial tree where laminar blood flow is disturbed. Upregulation of adhesion molecules in response to turbulent blood flow or other proinflammatory stimuli is an important feature of the disease.[6] Hence, circulating levels of soluble adhesion molecules such as ICAM-1, VCAM-1, E-selectin, and P-selectin may reflect the inflammatory response of the endothelium. In the present study, we did not however find any changes in the levels of these circulating adhesion molecules after 12 weeks of INP treatment. In a previous study investigating the effects of SET on endothelium-derived inflammatory markers and walking capacity in patients with IC, a significant increase in walking capacity and a significant reduction in E-selectin and ICAM-1 were observed after 8 weeks.[10] The results from the present study indicate that INP treatment of one leg does not affect the total vascular inflammatory burden caused by atherosclerosis, in contrast to what is observed after a period with SET in patients with IC. It is therefore likely that the improvement in walking capacity observed after SET in patients with IC is related both to positive systemic effects and to local effects of exercise.

Study limitations

There are some limitations in the present study. We did not find any significant between-group differences in the change of the levels of any of the measured biomarkers after 12 weeks of treatment. However, this exploratory study of secondary outcome measures may have been underpowered to detect such between-group differences. Hence, the change in vWF after long-term INP treatment that was observed in the present study should be verified in a larger trial. The patients were instructed to treat only their most limiting leg throughout the 12-week period. As atherosclerosis is a systemic disease, INP treatment of one leg may not have been

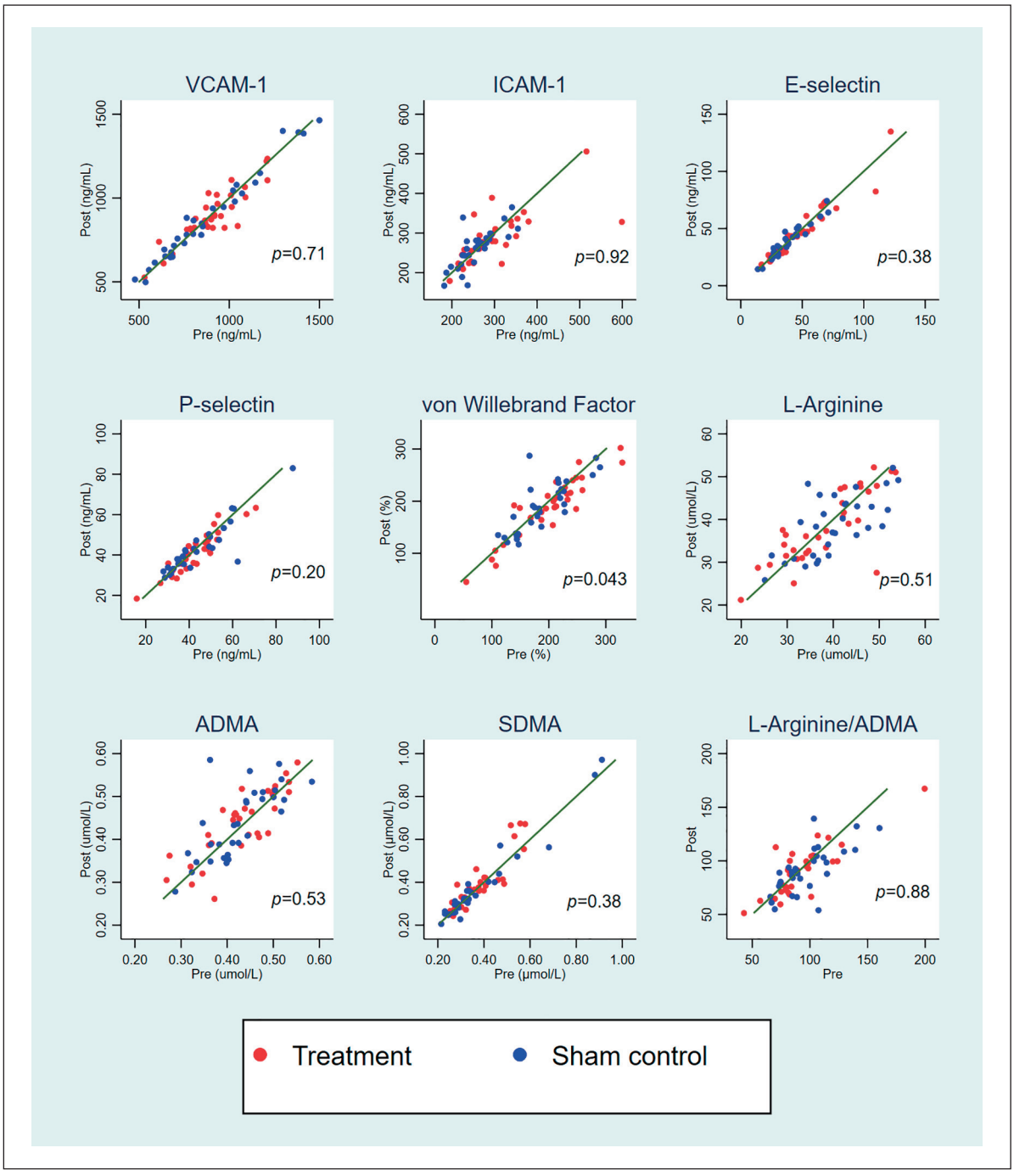

Figure 2. Concentrations of vascular biomarkers at baseline and after 12 weeks of intermittent negative pressure treatment.
Reference lines indicating post-values = pre-values. The *p*-values refer to χ^2 tests of proportions of patients with values increased versus decreased after 12 weeks.
ADMA, asymmetric dimethylarginine; ICAM-1, intracellular adhesion molecule-1; SDMA, symmetric dimethylarginine; VCAM-1, vascular adhesion molecule-1.

sufficient to affect the levels of the measured biomarkers enough to show between-group effects, especially as the biomarkers are not specific to PAD.

Conclusion

In this randomized controlled trial of patients with IC, there were no significant differences in the change in circulating levels of VCAM-1, ICAM-1, E-selectin, P-selectin, vWF, L-arginine, ADMA, and SDMA after treatment with –40 mmHg INP for 1 hour twice daily for 12 weeks, compared with sham treatment. However, a significantly larger proportion of the patients in the treatment group had a reduction in vWF compared with the sham control group, and the concentration of vWF was significantly reduced within the treatment group after 12 weeks, which might indicate a beneficial effect of INP treatment on endothelial activation and endothelial injury.

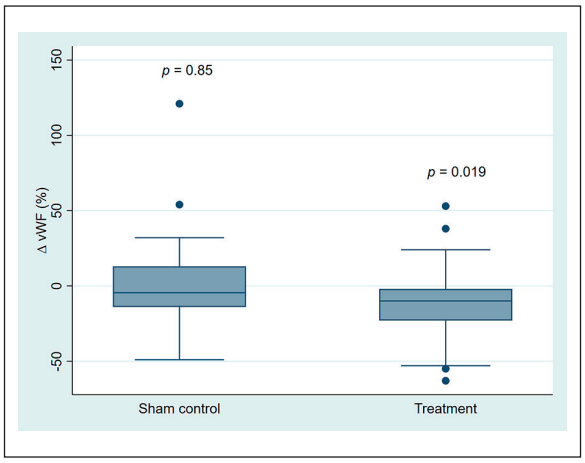

Figure 3. Box plot of changes in concentrations of vWF after 12 weeks of intermittent negative pressure treatment.
The *p*-values are for within-group changes (paired sample *t*-test).
vWF, von Willebrand factor.

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Declaration of conflicting interests

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Data accessibility

The biomarkers dataset is available upon request.

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IV

ELSEVIER

Clinical Research, Basic Science

Lower Extremity Intermittent Negative Pressure for Intermittent Claudication. Follow-Up after 24 Weeks of Treatment

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Background: Treatment with lower extremity intermittent negative pressure (INP) of -40 mm Hg for one hour twice daily for 12 weeks, increases walking capacity in patients with intermittent claudication (IC). However, the effects of INP treatment beyond 12 weeks have not been elucidated. The aim of the present study was to investigate the clinical effects of INP treatment after 24 weeks in patients with IC.

Methods: This was a follow-up study after a randomized sham-controlled trial, where patients randomized to the active treatment group were offered to continue treatment for 12 additional weeks (24 weeks in total). Treatment with -40 mm Hg INP was applied in a pressure chamber sealed around the lower leg, and the patients were instructed to treat themselves at home one hour in the morning and one hour in the evening. Pain free walking distance (PWD), maximal walking distance (MWD), resting ankle-brachial index (ABI) and post exercise ABI were measured at baseline, after 12 and 24 weeks.

Results: Ten out of 32 patients (31%) from the active treatment group in the initial trial were included in this follow-up study. At baseline, PWD was (mean ±SD) 151 ± 91 m and MWD was 362 ±159 m. There was a significant increase in both PWD and MWD after 24 weeks of treatment, compared to baseline (ANOVA; P= 0.006 and P= 0.012, respectively). Post hoc tests revealed that PWD increased significantly from baseline to 12 weeks (mean 81 m; 95% CI [6, 156]; P = 0.032), and that MWD increased significantly from 12 to 24 weeks (mean 145 m; 95% CI [22, 268]; P = 0.018). There were no significant changes in resting ABI or post exercise ABI during the 24-week treatment period (ANOVA; P= 0.157 and P= 0.450, respectively).

Conclusion: Both PWD and MWD improved after treatment with – 40 mm Hg INP for one hour twice daily for 24 weeks, compared to baseline. The main improvement in PWD occurred during the first 12 weeks of treatment, whereas the main improvement in MWD occurred between 12 and 24 weeks of treatment.

Declaration of Conflicting Interests: HH is employed by Otivio AS with funding from The Research Council of Norway. IM is the CSO and shareholder in Otivio AS. Otivio AS has the commercial rights to the INP technology used in this study. None of the other authors have any competing interests, financial or otherwise. The authors alone are responsible for the content and writing of the paper.

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INTRODUCTION

Peripheral artery disease (PAD) is associated with significant morbidity, increased mortality, and decreased quality of life. About one third of the patients diagnosed with PAD suffers from intermittent claudication (IC).[1] Management of cardiovascular risk factors including pharmacological secondary prevention, and supervised exercise therapy (SET) are the first line treatments for patients with IC.[2] The purpose is to lower the risk of cardiovascular events, and to improve, stabilize or slow down the progression of leg symptoms. However, as the availability and adherence to SET programs are low,[3,4] other treatment modalities have been suggested.[5]

Lower extremity intermittent negative pressure (INP) treatment increases blood flow, reduces leg symptoms and has been suggested as a treatment option for patients with PAD in several studies.[6-12] In a recent double-blind randomized sham-controlled trial from our research group, we found that treatment with lower extremity INP one hour twice daily for 12 weeks increased walking capacity in patients with IC compared to sham treatment.[12] However, the potential clinical effects of continued INP treatment beyond 12 weeks have not been elucidated. The aim of the present study was therefore to investigate the clinical effects of INP treatment after 24 weeks in patients with IC. We hypothesized that patients with IC would continue to improve walking capacity from 12 to 24 weeks of INP treatment.

METHODS

Participants

The present study was a follow-up after a randomized controlled trial investigating the clinical effects of INP treatment for one hour, twice daily for 12 weeks in 63 patients with IC (active treatment n=32, sham treatment n=31).[12] After 12 weeks of treatment, patients in the active treatment group receiving -40 mmHg INP were offered to continue treatment for 12 additional weeks (24 weeks in total). The patients were instructed to treat themselves at home one hour in the morning and one hour in the evening. The patients' most symptomatic leg identified after a treadmill test at baseline was chosen as the leg to be treated.

INP Treatment

INP of -40 mmHg was applied in a pressure chamber sealed around the patient's lower leg in cycles of 10 s negative pressure and 7 s atmospheric pressure generated by a pumping device, as previously described[12] (Fig. 1).

Fig 1. Device for lower extremity intermittent negative pressure treatment. Intermittent negative pressure is generated in a pressure chamber sealed around the patient's lower leg by a pump unit that is removing air from and venting the pressure chamber.

Variables

Background variables were recorded based on a standardized registration form. Pain free walking distance (PWD), maximal walking distance (MWD), resting ankle-brachial index (ABI), and post exercise ABI were measured at baseline, at 12 weeks and at 24 weeks of INP treatment. ABI was measured according to the guidelines from The American Heart Association.[13] PWD and MWD were measured on treadmill with a ramp protocol.[14] Post exercise ABI was measured within one minute after the end of the treadmill test.

Statistics

Continuous variables are presented as mean ±standard deviation and categorical variables as number (%). Normality was assessed with histograms, qq-plots, and residual plots. Repeated measures analysis of variance (ANOVA) was performed to determine if there were changes in PWD, MWD, resting ABI and post exercise ABI over the 24-week treatment period. The assumption of sphericity was assessed with Mauchly's test. Bonferroni correction was performed for post hoc comparisons of baseline vs. 12 weeks and 12 weeks vs. 24 weeks. P-values <0.05 were considered statistically significant. All analyses were performed

Table I. Patient's characteristics at baseline

Age (years)	71 ±7
Male sex	7 (70)
Body Mass Index (kg/m^2)	26.5 ±2.8
Smoking	
Current	3 (30)
Previous	5 (50)
Never	2 (20)
Diabetes mellitus	4 (40)
Chronic renal failure	0 (0)
Hypertension	9 (90)
Hypercholesterolemia	9 (90)
Coronary artery disease	5 (50)
Cerebrovascular disease	2 (20)
Antiplatelet agent	9 (90)
Anticoagulant agent	2 (20)
Statin	9 (90)
Antihypertensive agent	9 (90)
Localization of disease	
Suprainguinal	2 (20)
Infrainguinal	6 (60)
Supra- and infrainguinal	2 (20)
Previous intervention in treated leg	6 (60)
Pain free walking distance (m)	151 ±91
Maximal walking distance (m)	362 ±159

Continuous variables are presented as mean ±standard deviation, categorical variables are presented as number (%).

using Stata version 16 (Stata Inc. North Station, TX, USA).

Ethics

The study was approved by the Regional Committee for Medical and Health Research Ethics in Norway (ref: 9006) and was a follow-up study after a recent randomized controlled trial (NCT03640676). Written informed consent was obtained from all patients before inclusion.

RESULTS

Of the 32 patients randomized to the active treatment group in the initial trial, 10 patients (31%) volunteered to continue treatment for 12 additional weeks (24 weeks in total) and were included in the present follow-up study. Mean age was 71 ± 7 years, and seven patients were men. Two patients had suprainguinal disease, six patients had infrainguinal disease, and two patients had both supra- and infrainguinal disease (Table I).

At baseline, PWD was 151 ± 91 m. A repeated measures ANOVA showed that 24 weeks of INP treatment had a statistically significant effect on PWD ($F(2,18)=6.95$; $P=0.006$) (Fig. 2). Post hoc tests revealed that PWD increased significantly from baseline to 12 weeks (mean 81 m; 95% CI [6, 156]; $P=0.032$), but there was no significant change in PWD from 12 to 24 weeks (mean 19 m; 95% CI [-56, 94]; $P=1.00$).

At baseline, MWD was 362 ± 159 m. For MWD, Mauchly's test indicated that the assumption of sphericity had been violated ($\chi^2(2)=8.86$; $P=0.012$), hence a repeated measures ANOVA with Greenhouse-Geisser correction ($\varepsilon=0.5989$) was performed, showing that 24 weeks of INP treatment had a statistically significant effect on MWD ($F(1.198,10.780)=8.55$; $P=0.012$) (Fig. 3). Post hoc tests showed no significant change in MWD from baseline to 12 weeks (mean 38 m; 95% CI [-85, 161]; $P=1.00$), but a significant increase in MWD from 12 to 24 weeks (mean 145 m; 95% CI [22, 268]; $P=0.018$).

At baseline, resting ABI was 0.53 ±0.12, and post exercise ABI was 0.28 ±0.12 . There were no significant effects of INP treatment on resting ABI or post exercise ABI during the 24- week treatment period ($F(2,18)=2.06$; $P=0.157$ and $F(2,11)=0.86$; $P=0.450$, respectively)

DISCUSSION

In the present study we found that patients with IC receiving treatment with -40 mm Hg INP twice daily for 24 weeks increased both pain free- and maximal walking distance, compared to baseline. The main increase in PWD occurred during the first 12 weeks of treatment, whereas the main increase in MWD occurred from 12 to 24 weeks of treatment.

Several studies have suggested clinical effects of INP treatment on walking distance in patients with IC.[9-11] In a recently published randomized controlled trial from our research group,[12] we found a significant effect of INP treatment on PWD compared to sham treatment after 12 weeks. Further, a subgroup analysis showed a significant effect on both the PWD and the MWD compared to sham treatment for the patients with the most symptomatic disease (baseline PWD<200 m).[12] The present follow-up study is to our knowledge the first study that describe clinical effects of INP treatment beyond 12 weeks.

In the present study, we observed an increase in PWD during the first 12 weeks, whereas MWD increased during the last 12 weeks of the 24-week treatment period. This may be explained by the fact that many patients with IC have a low exercise capacity due to concomitant heart and lung diseases, which in addition to the leg pain, may restrict their MWD. An initial improvement in PWD may allow

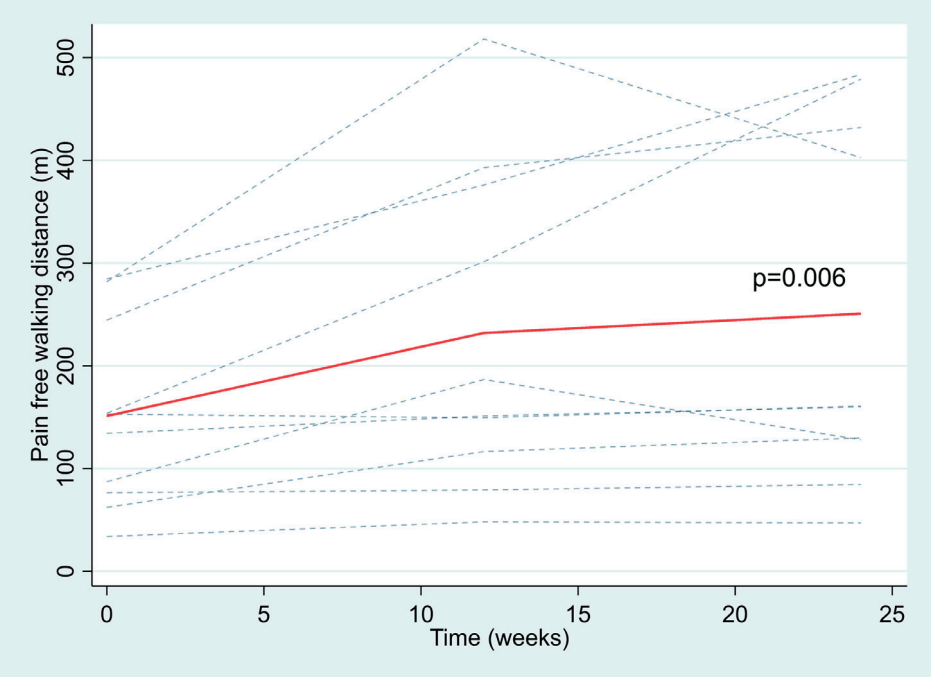

Fig. 2. Pain free walking distance plotted at baseline, 12 weeks, and 24 weeks of treatment with lower extremity intermittent negative pressure. Blue dashed lines represent individual patients ($n = 10$), red line represents the mean values at each time point. Overall P-value for repeated measures ANOVA is presented.

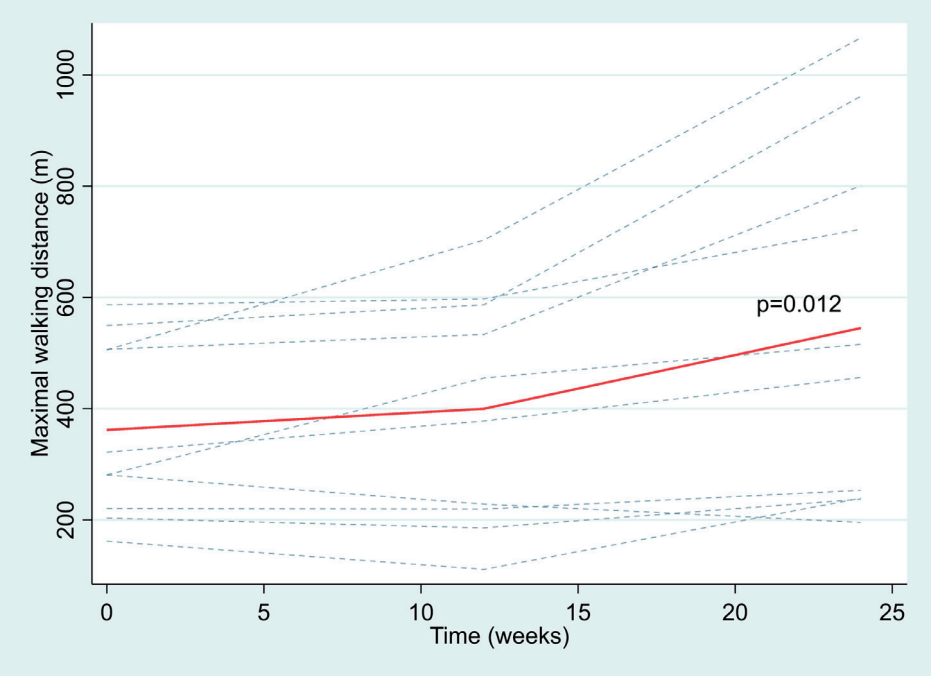

Fig. 3. Maximal walking distance plotted at baseline, 12 weeks, and 24 weeks of treatment with lower extremity intermittent negative pressure. Blue dashed lines represent individual patients ($n = 10$), red line represents the mean values at each time point. Overall *P*-value for repeated measures ANOVA is presented.

for more physical activity, which in turn improves the exercise capacity and the MWD. Participation in SET programs is shown to be effective and recommended for the treatment of IC[2], but is limited by poor adherence and low availability.[3,4] One reason for the poor adherence might be that the patients are exposed to pain during the exercise. The improvement in PWD obtained during 12 weeks of INP treatment may be sufficient to increase the adherence to SET, but the effects on MWD which seems to occur somewhat later also suggests that INP treatment could be a valuable supplement for IC patients when SET is unavailable, for example in rural areas.

The mechanisms of INP treatment resulting in long lasting effects in patients with PAD is not fully understood. However, it is shown that application of INP acutely increases arterial and skin blood flow.[15,16] The fluctuations in arterial flow promoted by INP leads to increased arterial shear stress, which induces flow-mediated vasodilation, and are thought to result in longer-lasting positive effects on the micro- and macro circulation in the treated extremity.[16,17] Hence, the improvement in walking capacity observed in patients with IC after long-term INP treatment can be interpreted as improved micro- and macro circulatory conditions in the treated extremity. This might also be applicable to patients with more advanced stages of PAD, as the underlying pathophysiology is the same. For patients with critical limb ischemia, endovascular or open surgical revascularization is the corner stone treatment, but have limitations related to patency, patient comorbidity and localization and extent of the disease. INP treatment could be an option for patients with critical limb ischemia not amenable for endovascular or open surgery, or as a supplement after endovascular or open surgical interventions with high risk of restenosis or graft occlusion. Whether INP treatment could contribute to limb salvage for patients with critical limb ischemia, or improve patency after endovascular or open surgical procedures should be subjects to further research.

In the present study, we did not observe any statistically significant increase in the PWD from 12 to 24 weeks of treatment. It might be that the main effect on PWD occurs during the first 12 weeks of treatment, however it may most likely be explained by lack of power due to the relatively low number of patients included in this follow-up study. Further, we did not observe changes in resting ABI or post exercise ABI during the study period. This is in line with studies investigating the effects of SET in patients with IC, showing increased walking capacity after SET without improvement in ABI.[18]

There are some limitations in this study. One patient peaked the treadmill test after 24 weeks of treatment, which probably have resulted in an underestimation of the treatment effect. The changes in walking distances observed in the present study are based on within group comparisons, without a control group. However, the effects on walking distances after the first 12 weeks are well documented in a double-blind randomized sham-controlled trial.[12]

CONCLUSION

In this follow-up study of 10 patients with IC, there were improvements in PWD and MWD after treatment with – 40 mmHg INP for one hour twice daily for 24 weeks. The improvement in PWD occurred during the first 12 weeks of treatment, whereas the improvement in MWD occurred between 12 and 24 weeks of treatment.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: HH, EMP, IM, AS, JH
Collected the data: HH, EMP, AS, JH
Performed the analysis: HH, LØH
Wrote the paper: HH
Revised the paper: HH, EMP, LØH, IM, AS, JH

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