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# Effects of remote ischaemic preconditioning on functional capacity and vascular and biochemical-metabolic biomarkers in patients with lower extremity artery disease

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# LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications that are referred to in the text by their Roman numerals (I–V):

I. Eerik, K., Kasepalu, T., Post, H., Eha, J., Kals, M., & Kals, J. (2025). Editor's choice – daily remote ischaemic preconditioning for intermittent claudication: A sham controlled randomised trial. *European Journal of Vascular and Endovascular Surgery*, *69*(2), 295–302. https://doi.org/10.1016/j.ejvs.2024.10.047

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III. Eerik, K., Kasepalu, T., Post, H., Eha, J., Kilk, K., Kals, M., & Kals, J. Effects of repeated remote ischaemic preconditioning on metabolic profiles of serum and skeletal muscle in patients with intermittent claudication: A sham controlled randomised trial. [Manuscript Submitted]

IV. Eerik, K., Kasepalu, T., Kuusik, K., Eha, J., Vähi, M., Kilk, K., Zilmer, M., & Kals, J. (2022). Effects of RIPC on the metabolome in patients undergoing vascular surgery: A randomized controlled trial. *Biomolecules, 12*(9), 1312. https://doi.org/10.3390/biom12091312

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**Author's contribution:**

Papers I–III and V: Participation in designing the study, recruiting participants and collecting the data, analysing the data and drafting the manuscripts.

Paper IV: Participation in analysing the data and drafting the manuscript.

# ABBREVIATIONS

| | |
|---|---|
| ABI | ankle-brachial index |
| ACEI | angiotensin-converting enzyme inhibitor |
| AI | augmentation index |
| AI@75 | heart rate corrected augmentation index |
| ANOVA | analysis of variance |
| ATP | adenosine triphosphate |
| BP | blood pressure |
| CAD | coronary artery disease |
| CI | confidence interval |
| CLTI | chronic limb-threatening ischaemia |
| CONSORT | Consolidated Standards of Reporting Trials |
| CVD | cardiovascular disease |
| GOLD | Global Initiative for Chronic Obstructive Lung Disease |
| eGFR | estimated glomerular filtration rate |
| ELISA | enzyme-linked immunosorbent assay |
| FDR | false discovery rate |
| FIA-MS/MS | flow injection analysis-tandem mass spectrometry |
| FMD | flow-mediated dilatation |
| GLP-1 | glucagon-like peptide-1 |
| HDL | high-density lipoprotein |
| HIF-1α | hypoxia-inducible factor-1α |
| HRV | heart rate variability |
| hs-CRP | high-sensitivity C-reactive protein |
| hs-TnT | high-sensitivity troponin T |
| IL | interleukin |
| IC | intermittent claudication |
| ICD | intermittent claudication distance |
| IQR | interquartile range |
| KIM-1 | kidney injury molecule 1 |
| LC-MS/MS | liquid chromatography-tandem mass spectrometry |
| LEAD | lower extremity artery disease |
| LDL | low-density lipoprotein |
| MACE | major adverse cardiovascular events |
| MALE | major adverse limb events |
| MWD | maximal walking distance |
| NGAL | neutrophil gelatinase-associated lipocalin |
| NO | nitric oxide |
| NT-proBNP | N-terminal pro B-type natriuretic peptide |
| NYHA | New York Heart Association |
| OS | oxidative stress |
| PAD | peripheral artery disease |
| PCSK9 | proprotein convertase subtilisin/kexin type 9 |

| | |
|---|---|
| PWV | pulse wave velocity |
| QoL | quality of life |
| RCT | randomised-controlled trial |
| RIC | remote ischaemic conditioning |
| RIPC | remote ischaemic preconditioning |
| RISK | reperfusion injury salvage kinase |
| SDF-1α | stromal-derived factor-1α |
| SET | supervised exercise therapy |
| SGLT-2 | sodium/glucose cotransporter 2 |
| TRC | time to relief from claudication |
| VascuQoL-6 | Vascular Quality of Life Questionnaire-6 |
| VEGF | vascular endothelial growth factor |
| WINPEPI | PEPI-for-Windows |

# 1. INTRODUCTION

Lower extremity artery disease (LEAD), also known as peripheral artery disease (PAD), is a manifestation of systemic atherosclerosis that results in narrowing or obstruction of the lower limb arteries and impaired blood supply to the legs (Gornik et al., 2024; Nordanstig et al., 2024). LEAD is a common cardiovascular condition and represents the third leading cause of atherosclerosis-related morbidity after coronary heart disease (CAD) and cerebrovascular disease. More than 230 million people worldwide are affected, and the global burden of LEAD has increased substantially over the recent decades (Eid et al., 2023).

IC is a common clinical manifestation of LEAD and is characterised by exercise-induced calf discomfort relieved by rest (Gornik et al., 2024; Nordanstig et al., 2024). Functional capacity and quality of life (QoL) in IC are largely determined by exertional leg pain. Consequently, symptom relief, improved mobility and improved QoL are central treatment goals, alongside the prevention of disease progression and reduction of cardiovascular and cerebrovascular events (Gornik et al., 2024; Nordanstig et al., 2024).

Conservative management of IC includes modification of risk factors, supervised exercise therapy (SET) and use of pharmacological agents to improve walking performance (Gornik et al., 2024; Nordanstig et al., 2024). Although SET is the most effective non-invasive treatment, it is frequently underutilised or unavailable and pharmacological options provide only modest benefit and are not suitable for all patients (Mazzolai et al., 2024). Patients with IC are frequently undertreated with guideline-directed medical therapy (Aboyans & Chastaingt, 2023), whereas revascularisation is typically reserved for advanced disease or refractory cases to conservative management (Gornik et al., 2024; Nordanstig et al., 2024). As a result, there is a substantial unmet need for novel, non-invasive therapies that can meaningfully improve walking performance and QoL in IC.

Remote ischaemic preconditioning (RIPC) is a phenomenon in which brief, non-injurious repeated episodes of ischaemia and reperfusion in one vascular bed induce systemic protective effects, rendering distant ischaemia-sensitive organs, like the heart, kidneys and skeletal muscle, more resistant to ischaemia-reperfusion injury (Heusch et al., 2015). This biological concept is particularly relevant to LEAD, including IC, which is characterised by endothelial dysfunction, microvascular impairment, inflammation, oxidative stress (OS) and skeletal muscle metabolic abnormalities (Hiatt et al., 2015). By enhancing tolerance to ischaemic stress and modulating vascular and muscular responses, RIPC represents a theoretically attractive therapeutic strategy.

A few studies have investigated the effects of RIPC in IC (Ahmed et al., 2019; Balin & Kivrak, 2019; Delagarde et al., 2015; Saes et al., 2013), but findings have been inconsistent and limited by inadequate sham control, insufficient verification of treatment adherence and lack of mechanistic assessment of RIPC effects. Moreover, the RIPC protocols have been heterogeneous, with most studies assessing a single RIPC episode rather than repeated RIPC, which may be more

effective in chronic conditions such as IC (Balin & Kivrak, 2019). Furthermore, it remains unclear whether repeated RIPC induces meaningful adaptation in IC, particularly because of the probability that the chronic limb ischaemia itself represents a state of background preconditioning.

Due to these uncertainties, the present study was designed to systematically evaluate the effects of repeated RIPC on walking performance and QoL in LEAD patients with IC, while simultaneously assessing vascular, biochemical and metabolic responses to better understand the mechanisms of RIPC.

# 2. REVIEW OF THE LITERATURE

## 2.1. Overview of lower extremity artery disease (LEAD) and intermittent claudication (IC)

### 2.1.1. Epidemiology and prognosis of LEAD

LEAD is characterised by narrowing or obstruction of the lower limb arteries, most commonly due to atherosclerosis (Gornik et al., 2024; Nordanstig et al., 2024). LEAD is a common cardiovascular condition with a growing global burden. It has been estimated that more than 230 million people worldwide are living with LEAD (Fowkes et al., 2013; Song et al., 2019). Owing mainly to population ageing, the prevalence of LEAD has increased substantially over recent decades, with approximately 45% more individuals being affected in 2015 compared with that in 2000 (Aday & Matsushita, 2021; Fowkes et al., 2013).

LEAD is associated with markedly increased mortality compared with individuals without that disease, with cardiovascular disease (CVD) representing the leading cause of death in patients with LEAD (Diehm et al., 2009). Symptomatic LEAD is associated with a higher mortality risk than that observed in asymptomatic disease (Bauersachs et al., 2020). The risk of CVD in LEAD correlates strongly with the disease severity and with both abnormally low and high ankle-brachial index (ABI), which remain independent predictors of mortality even after adjustment for traditional risk factors, such as advanced age, male sex, diabetes, hypertension, high total cholesterol and poor renal function (Resnick et al., 2004). Mortality risk is further amplified in patients with polyvascular disease, reflecting the presence of diffuse and advanced systemic atherosclerosis (Aday & Matsushita, 2021). Although patients with advanced presentations, such as chronic limb-threatening ischaemia (CLTI) or acute limb ischaemia, experience the highest risks of death, disability and amputation, IC is far from being a benign condition (Howard et al., 2015). Long-term all-cause mortality among patients with IC approaches approximately 30%, 50% and 70% at 5, 10 and 15 years, respectively (Norgren et al., 2007). CAD accounts for 40–60% of deaths in this population, while cerebrovascular disease and other vascular causes further contribute to mortality (Norgren et al., 2007).

From a limb-related perspective, disease progression in IC is relatively infrequent. Approximately 25% of patients experience clinical deterioration, most commonly within the first year after diagnosis (7–9% during the first year compared with 2–3% per year thereafter) (Abdulhannan et al., 2012). Major amputation is uncommon in IC, occurring in only 1–3% of patients over five years, however, limbs with ulceration due to arterial insufficiency treated without revascularisation will have a 19% risk of amputation at six months and 23% risk at one year (Abdulhannan et al., 2012).

Although IC is often stable from the limb's perspective, it confers a profound and sustained risk of cardiovascular mortality. This underscores the importance of aggressive medical treatment to reduce cardiovascular risk. Recent data

confirm a reduction in mortality among patients with LEAD who receive guideline-directed medical therapy (Fowler et al., 2024).

### 2.1.2. Functional limitations and quality of life impairment in IC

Symptoms of LEAD arise from progressive atherosclerosis leading to chronic arterial stenosis and occlusion (Hiatt et al., 2015). However, a large number of patients with LEAD are asymptomatic, accounting for 20–50% of cases (McDermott et al., 2008; Nordanstig et al., 2024). The clinical presentation of LEAD ranges from asymptomatic disease and chronic symptomatic disease, including IC, to CLTI and acute limb ischaemia. To categorise this wide range of presentations, several classification systems have been developed and remain widely used in routine clinical practice. The earliest of these, the Fontaine classification, is based solely on clinical symptomatology, whereas the Rutherford classification adds additional objective characteristics determined by non-invasive methods (Table 1) (Nordanstig et al., 2024).

IC is a common clinical manifestation of LEAD and is classically defined by exercise-induced calf discomfort that is relieved by rest (Gornik et al., 2024; Nordanstig et al., 2024). In patients with IC, exertional leg pain restricts mobility, representing a major determinant of functional capacity (Bauersachs et al., 2020). It is noteworthy that functional impairment is not only associated with the presence of symptoms. Asymptomatic individuals with LEAD demonstrate greater functional impairment, poorer QoL and more adverse calf muscle characteristics, such as smaller calf muscle area, higher calf muscle fat percent, poorer 6-minute walk performance and slower usual- and fast-paced walking speed, compared with patients with IC and sedentary, age-matched individuals without LEAD (McDermott et al., 2001; McDermott et al., 2008). These findings indicate that the absence of classical claudication symptoms does not mean preserved physical function (McDermott et al., 2001; McDermott et al., 2008). Furthermore, objective measures of physical performance and patient-reported symptoms often diverge, highlighting the complexity of functional limitation in chronic symptomatic LEAD.

Multiple mechanisms contribute to impaired physical function and reduced QoL in IC. These include coexisting CVD and other comorbidities that may limit physical performance and overall well-being. Nevertheless, the negative impact of LEAD, including IC, on physical components of QoL appears to be independent of other CVD (Regensteiner et al., 2008; Wu et al., 2017). Lower ABI values are associated with reduced vitality, depressive symptoms and feelings of hopelessness (Wu et al., 2017). Beyond physical impairment, LEAD, including IC, is also associated with reduced independence, social participation and autonomy, further contributing to diminished QoL (Abaraogu et al., 2018).

Depression is common in patients with LEAD and represents an important determinant of both functional status and clinical outcomes. A recent meta-analysis reported a pooled prevalence of depression of approximately 13% in patients with LEAD. It demonstrated an association with greater medical comorbidity and

a 20% increased risk of major adverse limb events (MALE) (Abi-Jaoudé et al., 2022).

In conclusion, these findings indicate that LEAD, including IC, is associated with substantial, multidimensional functional and psychosocial limitations that extend well beyond the walking impairment alone.

**Table 1.** Classification of the clinical forms of lower extremity artery disease according to the Fontaine and Rutherford classifications. Adapted from (Nordanstig et al., 2024).

| | ***Symptoms*** | ***Rutherford classification*** | ***Fontaine classification*** |
|---|---|---|---|
| | Asymptomatic | Category 0 | Stage I |
| ***Intermittent claudication*** | Mild claudication | Category 1 | Stage IIA: claudication at a distance ≥ 200 m |
| | Moderate claudication | Category2 | Stage IIB: claudication at a distance < 200 m |
| | Severe claudication | Category3 | |
| ***Chronic limb-threatening ischaemia*** | Ischaemic rest pain | Category4 | Stage III |
| | Minor tissue loss – non-healing ulcer, focal gangrene with diffuse pedal ischaemia | Category5 | Stage IV |
| | Major tissue loss – extending above transmetatarsal level, functional foot no longer salvageable | Category6 | |

### 2.1.3. Pathophysiology and risk factors of LEAD

**Pathophysiology of LEAD**

Arterial narrowing and obstruction reduce blood flow to the lower limbs and contribute to exercise impairment in LEAD. In addition to fixed haemodynamic obstruction, repeated episodes of exercise-induced ischaemia followed by reperfusion initiate downstream pathophysiological processes in the skeletal muscle (Hiatt et al., 2015; McDermott et al., 2020).

Under normal conditions, arterial blood flow is laminar, with minimal loss of pressure from the heart to the distal circulation (Hiatt et al., 2015). In the presence of arterial stenoses, pressure and flow decrease across the lesion. In the lower extremities, serial stenoses of vessels, such as the external iliac, superficial femoral and popliteal arteries, additively increase resistance, resulting in cumulative reductions in distal perfusion pressure (Hiatt et al., 2015). This integrated haemodynamic burden is reflected by ABI, which represents the net pressure loss

between the central circulation and the ankle. In patients with compressible arteries, LEAD is typically associated with a resting ABI <0.90 (Hiatt et al., 2015). However, most patients remain asymptomatic at rest, as skeletal muscle oxygen demand is low and resting calf muscle blood flow is comparable to that of age-matched controls due to compensatory reductions in the peripheral vascular resistance (Sørlie & Myhre, 1978).

During exercise, healthy individuals exhibit a marked increase in limb blood flow that tightly couples pulmonary oxygen uptake, cardiovascular delivery and mitochondrial oxygen utilisation. In LEAD, this coupling is disrupted by flow-limiting arterial stenoses, resulting in a blunted hyperaemic response and an early plateau in limb blood flow during the rest-to-exercise transition (Hiatt et al., 2015). During exercise, optimising muscle blood flow depends on vasodilation in the limb to minimise overall resistance to blood flow delivery to active muscle. Patients with LEAD have significantly impaired endothelium-dependent vasodilatation (Kals et al., 2006; Liao et al., 1991). OS contributes to endothelial dysfunction by increased generation of superoxide anion and related reactive oxygen species, which impair nitric oxide (NO)-mediated vasodilatation (Brevetti et al., 2008). In addition, exercise-induced release of endothelin-1, a potent vasoconstrictor, further opposes vasodilatation in skeletal muscles during increased metabolic demand (Mangiafico et al., 2000). Consistent with these mechanisms, endothelial dysfunction, as assessed by flow-mediated dilatation (FMD), correlates with the severity of LEAD (Grenon et al., 2014).

Although reduced exercise blood flow is central to exercise limitation, the weak relationship between haemodynamic impairment and functional capacity indicates that additional mechanisms contribute to exercise intolerance (Johnston et al., 2019). Recurrent exercise-induced ischaemia-reperfusion triggers systemic inflammatory activation and endothelial dysfunction, which are inversely associated with walking performance and functional capacity (Brevetti et al., 2010). Elevated inflammatory markers correlate with reduced mobility and accelerated functional decline in LEAD (McDermott et al., 2003; Nylaende et al., 2006). Concurrently, mitochondrial generation of reactive oxygen species during ischaemia-reperfusion leads to oxidative damage to muscle macromolecules and mitochondrial DNA (Melov et al., 1995). Evidence of the injury to mitochondrial DNA in both ischaemic and non-ischaemic limbs suggests that LEAD is associated with a systemic inflammatory and oxidative response rather than isolated local muscle ischaemia (Bhat et al., 1999).

In addition to vascular and inflammatory abnormalities, several structural and functional skeletal muscle changes have been identified in LEAD. These include muscle apoptosis and atrophy, fibre type switching, altered myosin heavy-chain expression and muscle fibre denervation (McDermott et al., 2020; Regensteiner et al., 1993). Such changes are partly mediated by elevated inflammatory signalling and contribute to the development of a distinct skeletal muscle myopathy in LEAD (McDermott et al., 2007; McDermott et al., 2020). In addition, adverse skeletal muscle characteristics, such as lower calf muscle density and weaker plantar flexion strength and knee extension power, are independently associated

with increased all-cause and cardiovascular mortality, even after adjustment for ABI (McDermott et al., 2012; McDermott et al., 2020).

At the metabolic level, LEAD disrupts the normal coupling between oxygen delivery and mitochondrial oxidative phosphorylation during exercise (Hiatt et al., 2015). Delayed activation of oxidative phosphorylation during the rest-to-exercise transition results in increased metabolic inertia and inefficient adenosine triphosphate (ATP) production. These abnormalities reflect impaired mitochondrial oxidative capacity, exacerbated by ischaemia-reperfusion-induced OS and electron transport chain dysfunction (Hiatt et al., 2015). Altered metabolism of muscle carnitine provides early evidence of impaired utilisation of oxidative substrate, with accumulation of acylcarnitines that reflects incomplete fatty acid oxidation (Hiatt et al., 1992). Elevated concentrations of plasma and skeletal muscle acylcarnitine, particularly in the ischaemic limbs, are inversely associated with exercise performance and predict functional limitation more strongly than does ABI (Hiatt et al., 1992). Concurrent elevations in muscle lactate further refer to the presence of impaired glucose oxidation, likely due to reduced electron transport chain flux and decreased pyruvate dehydrogenase activity (Barker et al., 2004).

**Risk factors**

LEAD shares traditional atherosclerotic risk factors with CAD and cerebrovascular disease, including advanced age, smoking and diabetes (Aday & Matsushita, 2021). A large meta-analysis identified age, current and former smoking and a history of CVD as the strongest risk factors for LEAD across high-, middle- and low-income countries (Fowkes et al., 2013). The association with sex is more complex: while the earlier studies suggested higher prevalence in men (Kannel & McGee, 1985), the more recent global data indicate a higher overall prevalence among women, with the between-sex differences varying by geographic region (Fowkes et al., 2013).

Marked racial disparities exist, with a higher prevalence of LEAD among Black individuals (Allison et al., 2007), whose lifetime risk is approximately 30% compared with that of 20% among non-Hispanic White individuals (Matsushita et al., 2019). In addition to traditional risk factors, emerging pathways, including chronic inflammation, thrombosis, enhanced platelet activation and microvascular disease, contribute to the risk of development of LEAD. Microvascular complications such as retinopathy and nephropathy, including microalbuminuria, are associated with increased risk of LEAD and reflect broader vascular vulnerability (Matsushita et al., 2017; Yang et al., 2020).

## 2.2. Current treatments for IC and their limitations

### 2.2.1. Lifestyle interventions and best medical therapy

Lifestyle interventions and optimal pharmacological therapy form the cornerstone of management in patients with LEAD and IC. The primary goals of medical treatment are to slow the progression of systemic atherosclerosis, reduce the incidence of major adverse cardiovascular events (MACE) and MALE, manage common comorbidities and improve QoL (Gornik et al., 2024; Nordanstig et al., 2024).

**Smoking cessation**

Smoking is strongly associated with LEAD severity, increased risk of amputation, graft occlusion and mortality (Young et al., 2019). Although smoking cessation has not been shown to improve walking distance directly, it significantly reduces cardiovascular risk and slows progression to CLTI (Abdulhannan et al., 2012). Combined interventions incorporating behavioural therapy, nicotine replacement and pharmacological support are more effective than single-modality approaches (Abdulhannan et al., 2012).

**Lifestyle modification and metabolic risk**

Patients with LEAD should be routinely screened for obesity, metabolic syndrome and diabetes (Nordanstig et al., 2024). A healthy lifestyle is associated with reduced risk of LEAD, with non-smoking, regular physical activity, moderate alcohol consumption and adherence to a Mediterranean diet showing inverse associations with the disease prevalence (Adegbola et al., 2022; Lopez-Laguna et al., 2018). Interestingly, normal body weight alone does not appear to confer protection, highlighting the importance of overall metabolic health rather than weight in isolation (Adegbola et al., 2022; Lopez-Laguna et al., 2018).

**Lipid control**

Strong evidence supports lipid-lowering therapy in reducing the risk of MACE, MALE and healthcare costs (Masson et al., 2022). The current guidelines classify LEAD as a very-high-risk condition and recommend intensive lipid-lowering therapy to a low-density lipoprotein (LDL)-cholesterol target of ≤1.4 mmol/L, preferably with high-intensity statins, with the addition of ezetimibe, if the target is not achieved (Nordanstig et al., 2024). Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) inhibitors provide additional reductions in the risk of MACE and MALE in high-risk patients. However, their use in IC is generally reserved for selected individuals who fail to reach lipid targets despite optimal therapy (Nordanstig et al., 2024).

**Hypertension control**

Hypertension contributes to atherosclerotic progression and the increased cardiovascular risk in LEAD (Aday & Matsushita, 2021). Large trials have demon-

strated reductions in mortality and cardiovascular events with BP lowering, particularly with angiotensin-converting enzyme inhibitors (ACEI) (Feringa et al., 2006; Ostergren et al., 2004). Antihypertensive therapy should be initiated in accordance with contemporary multisocietal guidelines (McEvoy et al., 2024; Nordanstig et al., 2024).

**Management of diabetes**

In patients with LEAD and type 2 diabetes, effective glycaemic control is an essential component of secondary prevention (Nordanstig et al., 2024). Metformin remains first-line therapy due to its favourable efficacy and safety profile (Cosentino et al., 2020; Palmer et al., 2016). Beyond glucose-lowering, glucagon-like peptide-1 (GLP-1) receptor agonists and sodium/glucose cotransporter 2 (SGLT-2) inhibitors provide cardiovascular and renal protection through largely glucose-independent mechanisms and are preferred in patients with both diabetes and CVD (Kristensen et al., 2019; McMurray et al., 2019). Although concerns regarding amputation risk were initially raised with canagliflozin (Neal et al., 2017), subsequent trials with empagliflozin and dapagliflozin did not confirm this association (Wiviott et al., 2019; Zinman et al., 2015), supporting cautious use of these agents in high-risk LEAD populations.

**Antiplatelet and antithrombotic therapy**

For patients with stable symptomatic LEAD, single antiplatelet therapy, preferably that with clopidogrel, is recommended to reduce cardiovascular risk (CAPRIE, 1996; Hiatt et al., 2017; Nordanstig et al., 2024). Routine dual antiplatelet therapy is not recommended outside specific post-procedural contexts (Nordanstig et al., 2024; Twine et al., 2023). In selected high-risk patients with acceptable bleeding risk, combination therapy with low-dose rivaroxaban and aspirin may be considered to further reduce MACE and MALE (Bonaca et al., 2020; Nordanstig et al., 2024; Twine et al., 2023).

**Undertreatment in clinical practice**

Despite the robust evidence and clear guideline recommendations, the majority of patients with LEAD do not receive optimal secondary prevention (Aboyans & Chastaingt, 2023). Although an established diagnosis of LEAD and often an accompanying significant burden of comorbidities, antiplatelet agents, heart rate-lowering agents, BP-lowering agents and lipid-lowering medications were prescribed in 63%, 34%, 46% and 45% of patients, respectively (Flu et al., 2010). Registry studies further indicate that patients with CLTI experience higher cardiovascular mortality than patients with myocardial infarction, yet remain undertreated despite comparable guideline-derived indications (Bager et al., 2023). Factors contributing to undertreatment include slightly different guidelines compared with those for CAD, underestimation of the severity of LEAD and low willingness to adhere to the guideline-based therapy by the patients themselves, due to significant comorbidities, depression and socioeconomic barriers (Aboyans & Chastaingt, 2023).

**Medications for symptom relief**

Pharmacological options specifically aimed at improving walking distance in IC are limited. Pentoxifylline, widely used in Europe, improves blood rheology and microcirculatory flow. However, meta-analyses indicate uncertain benefit, with low-certainty evidence for modest improvements in walking distance and no consistent effects on ABI or QoL (Broderick et al., 2020). Naftidrofuryl, also commonly used in Europe, acts as a vasodilator and improves cellular oxidative metabolism, demonstrating a clinically meaningful, albeit moderate, effect on improving walking distance (de Backer et al., 2012). Cilostazol, a phosphodiesterase-3 inhibitor, has demonstrated modest improvements in MWD and IC; however, it is associated with higher rates of adverse effects, such as headache (Brown et al., 2021). Currently, only naftidrofuryl or cilostazol can be considered for patients with lifestyle-limiting IC, who adhere to best medical therapy in Europe (Nordanstig et al., 2024), while only cilostazol has been available for years in the United States of America (Gornik et al., 2024). Importantly, there is no evidence that these agents improve the prognosis of LEAD (Gornik et al., 2024; Nordanstig et al., 2024).

### 2.2.2. Supervised exercise therapy and mechanisms of benefit

Current guidelines strongly recommend SET as a first-line treatment for walking impairment in IC (Gornik et al., 2024; Mazzolai et al., 2024; Nordanstig et al., 2024). The results of clinical trials show improvements in walking capacity by approximately 50–200% following SET (Abdulhannan et al., 2012). A large meta-analysis demonstrated a weighted mean improvement in maximal walking distance (MWD) of 180 m (95% confidence interval [CI], 130–230 m) and in IC distance (ICD) of 128 m (95% CI, 92–165 m) compared with the non-intervention group (Fakhry et al., 2012).

Most SET protocols involve supervised treadmill walking three times per week for approximately 30 minutes per session, typically to near-maximal claudication pain, for a minimum of 6 months (Thangada & McDermott, 2024). Supervision is considered essential for achieving optimal outcomes (Abdulhannan et al., 2012). However, considerable uncertainty remains regarding the optimal intensity, duration and content of exercise programmes. Meta-analyses have not identified any specific exercise component that could be independently predictive of improvements in MWD or ICD (Fakhry et al., 2012).

Despite its proven efficacy, participation in SET remains low (Mazzolai et al., 2024). Barriers include limited availability of SET programmes in many countries (including Estonia), a lack of physician referral and inadequate reimbursement or insurance coverage (Mazzolai et al., 2024; Thangada & McDermott, 2024). Additional patient-related factors include limited symptoms during formal treadmill testing, lack of motivation, comorbid conditions that limit the ability to exercise, travel burden, competing medical treatments and a perceived lack of benefit (Harwood et al., 2016). Even among enrolled patients, adherence and completion rates are often suboptimal, particularly in programmes of longer duration, higher

frequency, or limited activity variety (Harwood et al., 2016; Thangada & McDermott, 2024).

To overcome these limitations, alternative exercise strategies have been explored. Simply advising patients to walk more is ineffective (Savage et al., 2001). However, structured home-based walking programmes have demonstrated meaningful benefits, similarly improving MWD and ICD as SET (Thangada et al., 2023). Alternative exercise modalities, such as upper- and lower-extremity ergometry performed twice weekly for six months, result in significant improvements in ICD, with comparable benefits between arm and leg ergometry (Thangada & McDermott, 2024; Zwierska et al., 2005).

The mechanisms by which SET improves walking performance in IC are incompletely understood and are likely multifactorial (Mazzolai et al., 2024). Exercise training has been shown to improve endothelial function, as assessed by FMD, suggesting enhanced vasodilatory capacity as one potential mechanism (Brendle et al., 2001; Januszek et al., 2014). SET also improves skeletal muscle metabolism and oxidative capacity, contributing to more efficient energy utilisation during exercise (Hamburg & Balady, 2011). SET is linked to upregulation of antioxidant defence systems, indicated by increased expression of superoxide dismutase, inducible NO synthase and thioredoxin following sustained exercise training (Oh-ishi et al., 1997; Sumida et al., 2004; Vassilakopoulos et al., 2003).

Additional benefits of SET include improvements in gait biomechanics (Schieber et al., 2020). Meta-analyses further suggest modest effects of SET on reducing blood pressure (BP) and LDL cholesterol, although no consistent improvements have been observed in other cardiovascular risk factors such as glycaemic control, body weight, or smoking behaviour (Jansen et al., 2019). While SET remains the cornerstone of conservative management for IC, its limited availability and suboptimal adherence underscore the need for adjunctive therapies to improve claudication.

### 2.2.3. Revascularisation strategies

Revascularisation is indicated in patients with IC who have lifestyle-limiting symptoms refractory to optimal medical therapy and SET (Nordanstig et al., 2024). It is also indicated in the presence of CLTI (rest pain or tissue loss), where the limb salvage becomes the primary therapeutic goal (Nordanstig et al., 2023). The outcomes of revascularisation are strongly influenced by the anatomical location, extent and complexity of arterial disease, including lesion length, distribution and number (Abdulhannan et al., 2012). Revascularisation is not considered a first-line strategy in IC, because SET results in treadmill walking performance that is similar or even superior to that achieved with revascularisation (Murphy et al., 2012). Additionally, revascularisation below the aorto-iliac segment is associated with poorer patency and a higher risk of restenosis, making the risk-benefit ratio unfavourable for patients without CLTI (Gornik et al., 2024; Nordanstig et al., 2024).

**Revascularisation in patients with suprainguinal disease (aortoiliac segment)**
In patients with IC caused by aortoiliac disease, endovascular therapy is recommended as the first-line revascularisation strategy. Aortoiliac angioplasty is associated with high initial technical and clinical success rates exceeding 90%, with reported 5-year primary patency rates of approximately 72–79% (Abdulhannan et al., 2012). Primary stenting may further improve technical success and reduce long-term failure compared with angioplasty alone, without increasing complication rates (Jongsma et al., 2020; Koeckerling et al., 2023; Nordanstig et al., 2024), although definitive conclusions are limited by the heterogeneity across studies.

Open surgical reconstruction, such as aortobifemoral bypass, offers superior long-term durability, with 5-year primary patency rates approaching 90–95% (Abdulhannan et al., 2012). However, this benefit comes at the cost of higher perioperative morbidity and mortality (Abdulhannan et al., 2012). Consequently, surgical revascularisation is generally reserved for patients with extensive or diffuse disease, long occlusions or failure of endovascular therapy, in accordance with the contemporary guideline recommendations (Nordanstig et al., 2024).

**Revascularisation in patients with infrainguinal disease (femoropopliteal segment)**
In patients with IC and infrainguinal disease, the evidence supporting revascularisation over the best medical therapy combined with SET remains limited. The current guidelines, therefore, advocate a conservative approach, with revascularisation considered only in carefully selected patients with severe, lifestyle-limiting symptoms and favourable anatomical characteristics (Nordanstig et al., 2024). The decision to proceed with infrainguinal intervention should be weighed against the degree of functional impairment, response to non-invasive therapies, comorbidities, procedural risks and expected durability, given ongoing uncertainty regarding sustained clinical benefit (Nordanstig et al., 2024).

**Revascularisation in patients with infrapopliteal disease**
Infrapopliteal disease is more relevant in the context of CLTI and, in isolation, is rarely a cause of IC. There is insufficient evidence to support revascularisation in this territory (Gornik et al., 2024).

**Amputation**
Amputation is rarely indicated in IC and is generally reserved for patients with non-reconstructible disease, extensive tissue loss, severe infection, or intractable pain when limb salvage is no longer feasible (Abdulhannan et al., 2012). Although amputation should not always be viewed as a failure and may even improve the QoL in carefully selected cases, it is associated with substantial morbidity and mortality. Approximately 30% of patients undergo contralateral limb amputation within two years, and up to 50% die within five years following major amputation, with CVD being the most prevalent cause of death (Abdulhannan et al., 2012). Accordingly, in patients with IC, every effort should be made to

optimise conservative management and limb-preserving strategies before considering irretrievable interventions.

## 2.3. Remote ischaemic preconditioning (RIPC): concept and mechanisms

### 2.3.1. Historical background and definition

Ischaemic preconditioning (IPC) refers to a phenomenon in which brief, non-lethal episodes of ischaemia confer protection against a subsequent, more severe ischaemia-reperfusion insult (Heusch et al., 2015; Murry et al., 1986). The concept of local IPC was first described in the mid-1980s in experimental models of myocardial infarction. In a canine study, Murry et al. (1986) demonstrated that four cycles of 5-minute coronary occlusion followed by reperfusion markedly reduced the size of infarction during a subsequent 40-minute coronary occlusion.

Subsequent animal studies expanded this concept by showing that ischaemia in one coronary artery territory could protect myocardium supplied by a different coronary artery, a phenomenon termed remote myocardial preconditioning (Przyklenk et al., 1993). The observation that ischaemia in a non-cardiac organ could similarly confer myocardial protection led to the development of the concept of RIPC (Gho et al., 1996). In 2002, the first clinically relevant large-animal (swine) model demonstrated that RIPC induced by repeated hind-limb tourniquet occlusions significantly reduced myocardial infarct size, providing key translational evidence for this protective strategy (Kharbanda et al., 2002).

### 2.3.2. Neural, humoral and systemic pathways involved in RIPC

#### 2.3.2.1. Neural signal transfer

Signal transduction from the remote organ in which RIPC is initiated to the target organ (e.g., heart) involves the somatosensory system, spinal cord and autonomic nervous system (Heusch et al., 2015). The conditioning stimulus most commonly arises from transient ischaemia-reperfusion in a limb, but can also be triggered by surgical trauma, pharmacological activation of sensory fibres (e.g., capsaicin, bradykinin, adenosine), or electrical peripheral nerve stimulation (Heusch et al., 2015). The critical role of sensory afferent signalling is supported by experimental observations that local anaesthesia, pharmacological sensory nerve blockade, or peripheral nerve transection abolish RIPC-induced cardioprotection (Heusch et al., 2015).

Evidence for a neuro-humoral signalling mechanism is provided by studies demonstrating that blood-derived dialysates collected after peripheral administration of adenosine or capsaicin, nerve stimulation, or RIPC can transfer cardioprotection to naïve recipient hearts (Heusch et al., 2015). The involvement of spinal reflex pathways is further supported by the loss of protection following

spinal cord transection at T7-T10 levels or intrathecal opioid receptor blockade, as well as by the observation that spinal cord stimulation at C8-T2 reduces infarct size (Heusch et al., 2015). The efferent limb of the RIPC signalling cascade appears to be mediated by the autonomic nervous system, as cardioprotection is abolished by ganglionic blockade and attenuated by α- and β-adrenergic antagonism, vagotomy or muscarinic blockade (Heusch et al., 2015).

#### 2.3.2.2. Humoral signal transfer

Humoral signal transfer has been demonstrated in multiple experimental models. Early studies showed that coronary effluent from preconditioned hearts conferred protection to naïve hearts (Dickson et al., 1999). The presence of a circulating cardioprotective factor following RIPC was subsequently confirmed in a swine transplantation model, where RIPC of the limb in the acceptor pig provided potent cardioprotection to the subsequently transplanted and denervated donor heart (Konstantinov et al., 2005). In an isolated rabbit heart model, plasma from remotely preconditioned animals was cardioprotective when perfused into an isolated naïve heart (Shimizu et al., 2009).

Several candidate humoral mediators have been proposed. NO and its metabolites have received particular attention, as transient limb ischaemia induces shear stress-dependent NO release and increases circulating nitrite concentrations with cardioprotective properties (Tota et al., 2010). Consistent with this, RIPC-induced protection is abolished in endothelial NO synthase-deficient animals and when nitrite is scavenged from conditioned plasma (Tota et al., 2010).

Additional candidate mediators include stromal-derived factor-1α (SDF-1α), which exerts cardioprotective effects via C-X-C chemokine receptor type 4 signalling (Takahashi et al., 2010). Circulating SDF-1α levels increase following limb ischaemia-reperfusion, and pharmacological inhibition of this pathway partially attenuates RIPC-induced protection (Davidson et al., 2013). More recently, microRNA-144 has emerged as a putative mediator, with experimental evidence demonstrating that its inhibition abolishes RIPC-induced cardioprotection, while exogenous administration confers both acute and delayed protection (Li et al., 2014). Despite compelling preclinical data, the translational relevance of microRNA-144 in human RIPC remains uncertain, highlighting a gap between experimental findings and clinical application (Przyklenk, 2014). In addition, depletion of exosomes from conditioned perfusates abolishes protection, implicating the role of extracellular vesicles in inter-organ signal transfer (Giricz et al., 2014; Lang & Kim, 2022).

#### 2.3.2.3. Systemic and myocardial signalling pathways

RIPC exerts systemic effects that modulate inflammatory and endothelial responses, including reduced expression of endothelial adhesion molecules, decreased leukocyte-endothelium interactions and downregulation of proinflammatory gene expression (Anttila et al., 2016). Proinflammatory cytokines such as tumour

necrosis factor-α, interleukin (IL)-1β and IL-6 contribute to ischaemic tolerance, as blockade of IL-1 signalling abolishes protection (Bhuiyan & Kim, 2010). Despite these insights, systemic signalling pathways remain incompletely defined in humans, where cardioprotective effects appear less consistent than in animal models (Heusch et al., 2015).

At the myocardial level, RIPC involves locally acting mediators including adenosine, bradykinin, IL-10 and SDF-1α (Heusch et al., 2015). Adenosine, primarily via A1 receptors, plays a key role by improving mitochondrial function and reducing oxidative stress during ischaemia-reperfusion. In delayed RIPC, IL-10 contributes to cardioprotection by activating pro-survival signalling pathways, including phosphorylation of protein kinase B (Akt) and endothelial NO synthase. Consistent with these observations, RIPC has been shown to activate the reperfusion injury salvage kinase (RISK) pathway. Additional mediators, such as hypoxia-inducible factor-1α (HIF-1α) and heme oxygenase-1, have also been implicated, although their contribution appears variable and inconsistent across studies (Heusch et al., 2015).

Mitochondria are central to RIPC-mediated protection, with preservation of mitochondrial respiration and inhibition of mitochondrial permeability transition pore opening identified as key mechanisms (Anttila et al., 2016; Heusch et al., 2015). This is supported by evidence linking aldehyde dehydrogenase-2 activity to cardioprotection and improved outcomes in coronary artery bypass graft patients receiving RIPC (Heusch et al., 2015). Downstream pathways include activation of ATP-sensitive potassium channels, protein kinase C and phosphoinositide 3-kinase/Akt signalling, which promote cellular survival and calcium homeostasis, although these mechanisms are not yet fully integrated into a unified framework and remain less clearly defined in humans (Bhuiyan & Kim, 2010; Heusch et al., 2015).

In summary, RIPC integrates complex, interdependent neural, humoral and systemic signalling pathways that converge on mitochondrial preservation and the attenuation of inflammation and OS. While these mechanisms are consistently demonstrated in experimental models, their reproducibility and magnitude in humans remain variable, resulting in the translational challenges that continue to limit clinical application of RIPC (Heusch et al., 2015).

### 2.3.3. Single episode versus repeated RIPC: the biological rationale

Historically, the effects of RIPC have been primarily investigated following a single conditioning episode (Gho et al., 1996; Kharbanda et al., 2002). However, increasing evidence suggests that repeated RIPC applications may induce distinct, more durable biological effects, particularly in chronic disease settings (Lang & Kim, 2022).

A single episode of RIPC induces two temporally distinct phases of protection: an early window that develops within minutes and lasts for several hours, and a delayed “second window” that emerges after 12–24 hours and is mediated by

transcription-dependent mechanisms (Heusch et al., 2015; Kharbanda et al., 2009; Loukogeorgakis et al., 2005). Single-episode RIPC has therefore been predominantly studied in clinical contexts characterised by a predictable, acute ischaemic insult, such as cardiac surgery or acute myocardial infarction, where its effects are largely transient and focused on short-term protection against ischaemia-reperfusion injury. In contrast, repeated RIPC extends beyond these classical windows and appears capable of inducing more sustained vascular, microvascular, autonomic and molecular adaptations (Lang & Kim, 2022). Studies examining repeated RIPC have typically employed protocols similar to those used for a single episode, most commonly consisting of 3–4 cycles of 5-minute upper limb arterial occlusion followed by 5-minute reperfusion, but have delivered these sessions daily or every other day over intervention periods ranging from one week to up to one year (Lang & Kim, 2022).

From a clinical perspective, defining the minimal effective "dose" of repeated RIPC required to maximise vascular benefits remains an important unanswered question. Although the optimal duration is unclear, comparable improvements in cutaneous endothelial function have been observed after one and two weeks of repeated RIPC, suggesting that early adaptations may occur rapidly (Kim et al., 2020). Similarly, FMD was not further enhanced by eight weeks compared with two weeks of RIPC administered every other day, indicating a potential plateau effect with longer interventions (Jones et al., 2015).

In patients with heart failure, one week of repeated RIPC increased coronary artery flow reserve by approximately 20% (Kono et al., 2014). Moreover, one to three weeks of repeated RIPC improved FMD or attenuated the decline in endothelial function following ischaemia-reperfusion injury in patients with type II diabetes (Maxwell et al., 2019), CAD (Liang et al., 2015) and stroke (Hyngstrom et al., 2020). Longer-term interventions exceeding six months have been associated with reduced recurrent stroke rates and favourable modulation of thrombolytic, inflammatory and coagulation markers, suggesting sustained systemic effects of repeated RIPC (Meng et al., 2012; Meng et al., 2015). Collectively, these studies support the concept that repeated RIPC improves cardiovascular and vascular function across different cardiovascular conditions.

**Sustained vascular and microvascular adaptations**

Repeated RIPC appears to induce lasting adaptations in conduit arteries and microvasculature. Daily exposure to unilateral RIPC for 7 days resulted in significant improvements in brachial artery FMD and cutaneous microcirculation with effects persisting beyond the cessation of the intervention and extending beyond the classical late-phase of protection (Jones et al., 2014). Notably, these improvements were observed not only in the conditioned limb but also in the contralateral arm, indicating a systemic vascular response (Jones et al., 2014).

Consistent with these findings, several studies have demonstrated improvements in microvascular function following repeated RIPC (Kim et al., 2020; Kim et al., 2025; Lang et al., 2019). Lang et al. (2019) reported augmentations in maximal vasodilatation of approximately 20–50% after seven days of RIPC with

mechanistic data suggesting that these effects are mainly independent of NO signalling, implicating alternative endothelium-derived mediators or endothelium-independent vascular adaptations. Preliminary evidence also suggests that repeated RIPC may lead to modest reductions in systemic BP, potentially mediated through improvements in systemic endothelial function (Jones et al., 2014; Madias, 2011).

**Autonomic nervous system modulation**

Repeated, but not single, RIPC appears capable of modulating autonomic nervous system balance. While a single RIPC session did not alter heart rate variability (HRV), two weeks of repeated RIPC induced HRV changes consistent with a shift toward enhanced parasympathetic activity (Gardner et al., 2020). In healthy adult men, RIPC that was applied prior to repeated sprint exercise accelerated short-term cardiac autonomic recovery, as reflected by faster heart rate recovery at 60 seconds post-exercise. However, no effects on longer-term autonomic recovery or energy metabolism were observed (Lopes et al., 2018).

**Distinct genomic and molecular responses to repeated RIPC**

Experimental studies suggest that the molecular and genomic responses to repeated RIPC differ fundamentally from those associated with classical single-episode or second-window preconditioning (Depre et al., 2010; Kim & Lang, 2022). While all models reduced infarct size, NO-dependent mechanisms predominated in classical preconditioning, whereas repetitive ischaemic episodes were associated with downregulation of genes related to oxidative metabolism and upregulation of stress-response pathways, including unfolded protein response, autophagy and nuclear factor-kappa B signalling (Depre et al., 2010).

**Angiogenesis and vascular remodelling**

Angiogenic mechanisms may further contribute to the adaptations observed with repeated RIPC (Lang & Kim, 2022). Kimura et al. (2007) demonstrated increased circulating vascular endothelial growth factor (VEGF) levels and mobilisation of endothelial progenitor cells following four weeks of repeated RIPC. VEGF has been shown to enhance myocardial functional recovery following ischaemia (Guzman et al., 2008). The ischaemic stimulus associated with RIPC activates HIF-1α, a transcriptional regulator that induces VEGF expression and multiple proteins involved in vasomotor regulation and angiogenesis (Albrecht et al., 2013; Cai et al., 2013).

## 2.4. Clinical evidence of RIPC in cardiovascular diseases

### 2.4.1. Acute and perioperative RIPC

RIPC has been most extensively studied in acute and perioperative cardiovascular settings, where episodes of ischaemia-reperfusion injury are more predictable and temporally defined. The principal clinical contexts include cardiac surgery, major vascular surgery and percutaneous coronary interventions. These settings are particularly attractive for evaluating RIPC because the timing of the intervention can be standardised, and RIPC's biological effects can be assessed using biochemical and clinical outcomes.

Early single-centre randomised trials in cardiac surgery suggested that RIPC confers perioperative myocardial protection, as reflected by lower postoperative levels of cardiac troponin (Candilio et al., 2015; Thielmann et al., 2013), and may reduce mortality (Thielmann et al., 2013). Similarly, the results of single-centre randomised-controlled trials (RCTs) in vascular surgery showed reductions in markers of myocardial and renal injury following RIPC (Ali et al., 2007; Kasepalu et al., 2020a; Kepler et al., 2020). These findings generated considerable enthusiasm and supported the concept that brief, remote ischaemic stimuli could provide systemic organ protection in humans. However, many of these early studies were limited by small sample sizes, heterogeneity of RIPC protocols and reliance on surrogate biochemical endpoints (such as cardiac troponin) rather than clinically meaningful outcomes such as MACE or mortality.

In contrast, subsequent larger multicentre randomised trials and meta-analyses failed to demonstrate consistent clinical benefit of a single episode of RIPC in cardiac (Benstoem et al., 2017; Hausenloy et al., 2015; Meybohm et al., 2015; Wang et al., 2017) and non-cardiac (de Freitas et al., 2019; Greco et al., 2025; Stather et al., 2019; Zhang et al., 2019) surgery. In these studies, conducted in populations receiving modern anaesthetic techniques and optimised cardioprotective pharmacotherapy, RIPC showed neutral effects on major clinical outcomes, including myocardial infarction, acute kidney injury and mortality. These findings raised questions regarding the translation of RIPC into routine clinical practice.

Several explanations have been proposed to account for the discrepancies between early positive studies and later neutral trials. These include interactions with anaesthetic agents, particularly volatile anaesthetics and propofol (Zangrillo et al., 2015), background use of cardioprotective medications (Zhou et al., 2013) and the possibility that advances in perioperative care already provide substantial endogenous protection, thereby limiting the incremental benefit of RIPC. Collectively, these observations suggest that RIPC's effectiveness is context-dependent and may be attenuated in settings with optimised baseline care.

Overall, the concept of RIPC has not been established in acute and perioperative settings. In addition, no clear benefit has been demonstrated in major subgroups defined by age, diabetes status or timing of interventions (Hausenloy et al., 2015; Hausenloy et al., 2019). This also raises the question of whether the

results of trials assessing the effect of a single episode of RIPC in acute settings can be extrapolated to chronic CVD, highlighting the need to evaluate repeated RIPC in chronic conditions.

### 2.4.2. Repeated RIPC in chronic cardiovascular conditions

In contrast to acute applications, repeated or daily RIPC has been proposed as a conditioning strategy for chronic CVD, aiming to induce cumulative biological adaptation rather than transient protection (Lang & Kim, 2022). Conceptually, this approach is analogous to exercise training, whereby repeated exposure to a physiological stressor leads to durable improvements in vascular and metabolic function (Capecchi et al., 1997; Thijssen et al., 2022).

Repeated RIPC has been explored in several chronic CVDs, including stable CAD (Guo et al., 2023; Liang et al., 2015; Zagidullin et al., 2016), cerebrovascular disease (Hou et al., 2022; Mi et al., 2016; Wang et al., 2017), heart failure (Pryds et al., 2017) and LEAD (Ahmed et al., 2019; Balin & Kivrak, 2019; Delagarde et al., 2015; Hansen et al., 2019; Saes et al., 2013). Some studies have reported favourable effects on surrogate outcomes, e.g. improvements in endothelial function (Liang et al., 2015), BP and arterial stiffness (Pryds et al., 2017; Zagidullin et al., 2016), microcirculatory function (Guo et al., 2023; Kono et al., 2014) and selected cardiac functional measures (Chen et al., 2018; Guo et al., 2023). However, these findings have been inconsistent and often derived from studies with small sample sizes, short follow-up periods and heterogeneous intervention protocols. Many studies lack adequate sham control and blinding. Given that many outcome measures in chronic conditions, such as exercise tolerance, symptom burden and QoL indices, are highly susceptible to placebo effects, the lack of rigorous blinding substantially limits interpretability.

Currently, only in the field of neurology have large, randomised trials with repeated RIPC in patients with ischaemic stroke demonstrated clinical benefit, with improved functional outcomes (Blauenfeldt et al., 2024; Chen et al., 2022; Hou et al., 2022). In other fields, there is a lack of high-quality RCTs on repeated RIPC.

## 2.5. RIPC and IC

### 2.5.1. Early studies evaluating RIPC in IC

Compared with other cardiovascular conditions, relatively few clinical studies have investigated the effects of RIPC in patients with IC. These early studies have primarily assessed the effects of RIPC on walking performance in IC. Several small trials have reported improvements in walking performance following RIPC, including increases in MWD (Balin & Kivrak, 2019) and ICD (Ahmed et al., 2019; Balin & Kivrak, 2019; Saes et al., 2013). However, the preserved effects of RIPC on MWD have been modest compared to those of SET (Balin & Kivrak, 2019). Some studies have also reported favourable effects on the ABI (Ahmed et

al., 2019; Shahvazian et al., 2017), whereas others have demonstrated no significant benefit of RIPC on functional or haemodynamic outcomes (Delagarde et al., 2015; Hansen et al., 2019). Overall, the available evidence is derived mainly from studies with heterogeneous designs, limited blinding, no assessment of adherence to the treatment and limited follow-up, resulting in inconclusive evidence base. In addition, most studies focused solely on clinical endpoints, without accompanying mechanistic assessments, raising the question of whether the improvement in walking performance is due to RIPC or non-specific effects, precluding meaningful interpretation of both positive and neutral findings. These limitations in earlier trials highlight the need for well-designed, comprehensive trials to assess the effects of repeated RIPC in IC.

### 2.5.2. RIPC in the context of chronic limb ischaemia: theoretical considerations

Although RIPC is biologically appealing, its application in IC raises specific theoretical challenges related to the pathophysiology of chronic limb ischaemia. Unlike acute ischaemic events, IC is characterised by recurrent, often daily episodes of transient ischaemia-reperfusion during walking. This chronic exposure may itself induce adaptive or maladaptive changes within microvasculature and skeletal muscle, fundamentally altering responsiveness to external conditioning stimuli (Whittaker & Przyklenk, 2014). One hypothesis is that spontaneous ischaemia-reperfusion episodes in the affected limb activate and upregulate endogenous protective pathways; on the contrary, the other theory suggests that excessive ischaemia-reperfusion exposure may result in loss of conditioning benefit and potentially adverse effects, such as damage to collagen, which could translate into negative clinical consequences (Hummitzsch et al., 2021; Whittaker & Przyklenk, 2014).

Additionally, spontaneous episodes of ischaemia-reperfusion in the ischaemic leg do not necessarily equal to RIPC. Currently, there is a consensus that RIPC requires a healthy limb to activate complex neural, humoral and systemic pathways (Heusch, 2015). Furthermore, ischaemia during exercise may not last 5 minutes, as is thought necessary in established RIPC protocols (Heusch, 2015). Brief episodes of ischaemia during walking may not be sufficient to activate preconditioning signals. Collectively, these considerations highlight the reasons why IC represents both an attractive and challenging target for RIPC and stress the need for rigorous sham-controlled trials with integrated mechanistic endpoints to determine whether repeated RIPC confers meaningful biological or clinical effects in patients with IC.

## 2.6. Mechanistic targets of RIPC relevant to IC

### 2.6.1. Arterial stiffness

Arterial stiffness is a well-established marker of vascular ageing and an independent predictor of cardiovascular morbidity and mortality (Laurent et al., 2006; Mitchell et al., 2010; Vasan et al., 2023; Vlachopoulos et al., 2019). The two major determinants of arterial stiffness are age and BP (Regnault et al., 2024). Increased arterial stiffness reflects cumulative structural and functional changes within the arterial wall, caused by endothelial dysfunction, inflammation, OS and alterations in extracellular matrix composition (Regnault et al., 2024; Shirwany & Zou, 2010).

The most widely used non-invasive surrogate measures of arterial stiffness are pulse wave velocity (PWV), defined as the speed at which the pressure wave propagates along the arterial tree, and augmentation index (AIx). AIx is a measure of enhancement (augmentation) of central aortic pressure by the reflected pulse wave and is defined as the difference between the second and first systolic peaks expressed as a percentage of pulse pressure (Laurent et al., 2006; Pilz et al., 2024). PWV and AIx capture systemic vascular properties that extend beyond focal arterial stenoses.

In patients with IC, arterial stiffness is frequently increased and only partially explained by traditional cardiovascular risk factors or ABI values (Zahner et al., 2017). Elevated arterial stiffness is associated with impaired endothelial function, reduced arterial compliance and adverse arterial wall remodelling, all of which may indirectly contribute to exercise intolerance and cardiovascular risk (Coutinho et al., 2011; Mendes-Pinto & Rodrigues-Machado, 2019).

RIPC has been proposed to influence arterial stiffness through endothelial-dependent mechanisms, including enhanced NO bioavailability and attenuation of OS (Ghimire et al., 2025; Heusch et al., 2015). Some small studies have reported favourable effects of a single episode of RIPC on arterial stiffness (Kuusik et al., 2019; Zagidullin et al., 2016). However, more recent meta-analysis has yielded neutral results (Amorim et al., 2025). Assessing the effects of RIPC on arterial stiffness in IC can provide a mechanistic insight into systemic vascular conditioning.

### 2.6.2. Oxidative stress, inflammation and end-organ injury

IC is characterised by repeated episodes of skeletal muscle ischaemia-reperfusion during walking, leading to increased reactive oxygen species production and activation of inflammatory pathways (Steven et al., 2017). This chronic oxidative and inflammatory burden contributes not only to local skeletal muscle dysfunction, but also to systemic endothelial impairment and progression of atherosclerotic disease (Steven et al., 2017).

RIPC has been shown to attenuate endothelial dysfunction. Brief cycles of forearm ischaemia-reperfusion conferred both immediate and delayed protection against endothelial dysfunction in the contralateral arm (Kharbanda et al., 2002;

Loukogeorgakis et al., 2005). Subsequent studies demonstrated that repeated RIPC improves endothelial function and microcirculatory responses in healthy individuals (Jones et al., 2014), with similar effects reported in patients with acute myocardial infarction undergoing percutaneous coronary intervention (Manchurov et al., 2014) and patients with heart failure (Kono et al., 2014). However, the extent to which these protective mechanisms can be sustained or translated to chronic ischaemic conditions, such as IC, remains uncertain.

Patients with IC are at high risk of cardiovascular events and mortality (Aday & Matsushita, 2021; Nordanstig et al., 2024) and frequently exhibit evidence of subclinical myocardial (Linnemann et al., 2013) and renal injury (Paraskevas et al., 2009). In LEAD, even minor elevations in cardiac troponins are associated with substantially increased risks of mortality, myocardial infarction and limb loss, independent of renal function (Linnemann et al., 2013; Vrsalovic et al., 2022).

Biomarkers of OS, inflammation and organ function provide important insights into subclinical organ damage in IC. Assessing the effects of RIPC on these markers may provide additional mechanistic context for interpreting clinical findings.

### 2.6.3. Skeletal muscle dysfunction and ischaemic tolerance

Functional limitations in IC cannot be explained solely by impaired limb perfusion. Skeletal muscle pathology plays an essential role in IC. Muscle atrophy and sarcopenia are common and worsen with increasing disease severity (McDermott et al., 2020). Structural abnormalities, such as fibrosis, fatty infiltration and microvascular rarefaction, are characteristic features of affected muscle (McDermott et al., 2020). In parallel, mitochondrial dysfunction and increased OS contribute to impaired energy metabolism and exercise intolerance (Pizzimenti et al., 2020).

These muscle-level abnormalities play a central role in determining walking performance, QoL, mobility loss and clinical outcomes in IC (Pizzimenti et al., 2020). Furthermore, skeletal muscle abnormalities help to explain why improvements in functional capacity may occur independently of changes in limb haemodynamics, highlighting the importance of improvement of skeletal muscle biology as a key therapeutic target behind the arterial revascularisation (Hamburg & Balady, 2011).

In animal models of skeletal muscle, RIPC has been proposed to enhance cellular tolerance to ischaemic stress by activating protective signalling pathways that preserve mitochondrial integrity and limit ischaemia-reperfusion injury (Eberlin et al., 2008; Kocman et al., 2015; Mansour et al., 2012; Park et al., 2016). Studies in healthy individuals have shown that RIPC delays fatigue during handgrip exercise (Barbosa et al., 2015), facilitates greater strength gains during strength training (Surkar et al., 2020) and influences positively the local neuromuscular performance (Niespodziński et al., 2021).

Given the central role of skeletal muscle abnormalities, such as fibrosis, fatty infiltration and microvascular rarefaction, in IC and RIPC's potential in enhancing skeletal muscle resilience to ischaemia-reperfusion injury, assessing skeletal muscle responses to repeated RIPC is critical for determining its effectiveness in IC.

### 2.6.4. Metabolic and mitochondrial adaptations

Metabolic dysfunction is a key feature of IC, reflecting impaired mitochondrial oxidative capacity, altered substrate utilisation and accumulation of metabolic intermediates (Pipinos et al., 2007). Abnormalities in amino acid metabolism, fatty acid oxidation and energy homeostasis have been reported in patients with LEAD and are associated with disease severity, prognosis and reduced ischaemic and exercise tolerance (Brass et al., 2004; Pipinos et al., 2007).

LEAD is characterised by systemic metabolic alterations that reflect localised skeletal muscle ischaemia. Reduced serum lysine promotes excessive plasmin activation, leading to enhanced proteolysis and vascular tissue degradation, a process exacerbated by elevated trimethylamine N-oxide, which further drives atherosclerosis (Azab et al., 2020). Metabolomic studies further demonstrate a distinct biochemical signature in IC, characterised by alterations in amino acid, lipid and energy metabolism pathways. In IC, there are elevations in short-chain acylcarnitines, including hydroxypropionylcarnitine and tiglylcarnitine, which indicate mitochondrial dysfunction and incomplete fatty acid oxidation, which may contribute to reduced exercise capacity (Ismaeel et al., 2019). Impaired energy metabolism is further evidenced by reduced creatine availability and elevated serum creatinine, reflecting diminished phosphocreatine stores and muscle dysfunction. Mitochondrial β-oxidation is disrupted, as shown by increased circulating carnitine and propionylcarnitine alongside reduced fatty acids, suggesting defective lipid utilisation in muscle tissue (Azab et al., 2020).

Compared to non-LEAD controls, IC patients exhibit reduced levels of histidine and ornithine, alongside an increased phenylalanine-to-tyrosine ratio, reflecting heightened oxidative stress, inflammation and impaired nitric oxide bioavailability due to disrupted arginine metabolism (Ismaeel et al., 2019). Alterations in lipid metabolism are also evident, with decreased levels of specific ceramides (e.g., Cer (43:1), Cer (44:0)) and changes in cholesteryl ester (CE) composition, particularly an increased CE (18:1)/CE (18:2) ratio (Ismaeel et al., 2019). Furthermore, LEAD is characterised by elevated triglycerides in both LDL and high-density lipoprotein (HDL) particles, lower proportions of polyunsaturated fatty acids and higher monounsaturated fatty acids (Tikkanen et al., 2021).

RIPC has the potential to modulate these metabolic disturbances, and its effects are thought to be mediated through changes in amino acid and energy metabolism. Experimental studies have shown that pathways involving alanine, aspartate, glutamate, arginine and proline may contribute to the beneficial effects of RIPC (Baranovicova et al., 2021; Chao de la Barca et al., 2016; Shen et al., 2017). In

animal models, RIPC partially normalises impaired energy metabolism and promotes a shift towards ketone body synthesis, potentially enhancing energy availability for ischaemia-sensitive organs such as the brain and the heart (Baranovicova et al., 2021). The ketone body 3-hydroxybutyrate has been shown to facilitate RIPC effects, as its levels normalise within 24 hours of ischaemia in RIPC-treated animals, in contrast to persistently low levels in non-RIPC animals (Baranovicova et al., 2021).

In addition to their role in energy metabolism, some amino acids mediate the effects of RIPC through other pathways. RIPC has been associated with increased carnosine levels, a molecule with well-established antioxidant properties (Boldyrev et al., 2013; Chao de la Barca et al., 2016). Following RIPC, higher levels of kynurenine have been detected, suggesting activation of the tryptophan-kynurenine pathway (Chao de la Barca et al., 2016). Kynurenine is a vasoactive metabolite capable of inducing coronary vasodilation and may contribute to RIPC-mediated cardioprotection (Wang et al., 2010). In addition to amino acids, lipid-related metabolites such as palmitic, stearic, oleic and linoleic acid can stimulate toll-like receptor 4 signalling and activate pro-inflammatory pathways (Shen et al., 2017; Shi et al., 2006). These metabolites are upregulated during ischaemia-reperfusion injury but are downregulated by RIPC, suggesting a potential anti-inflammatory effect (Shen et al., 2017).

Patients with LEAD exhibit mitochondrial alterations and impaired function (Gonzalez-Freire et al., 2020; Pipinos et al., 2000). Mitochondria are a central target of RIPC-mediated protection, and experimental studies have shown that RIPC can preserve mitochondrial function, reduce oxidative damage and improve ATP production efficiency during subsequent ischaemic stress (Crestanello et al., 2002; Kobara et al., 1996; Quarrie et al., 2011).

Metabolomic profiling offers a comprehensive approach to assessing these processes by capturing integrated metabolic responses at both systemic and tissue levels. Therefore, evaluating the effects of repeated RIPC on both serum and skeletal muscle metabolome may provide important insights into metabolic and mitochondrial adaptations underlying functional outcomes in IC.

## 2.7. Methodological challenges in the research involving RIPC and resulting knowledge gaps

Several important methodological challenges have limited research on RIPC in IC. Previous studies have employed highly heterogeneous RIPC protocols, varying in the number and duration of ischaemia-reperfusion cycles, length of intervention and the mode of application.

Standardisation and verification of RIPC delivery represent additional challenges, especially in outpatient settings. Some studies have relied on conventional BP cuffs that patients applied themselves, potentially compromising both the integrity of the RIPC protocol and the effectiveness of blinding. Sometimes, objective confirmation of arterial occlusion and reporting of treatment adherence have

often been lacking, making it difficult to ascertain whether true episodes of ischaemia have consistently been delivered.

In addition, adequate sham control has often been absent or insufficiently described in previous trials. Given that walking performance and symptom perception in IC are highly susceptible to placebo effects and learning phenomena, the lack of rigorous blinding may have led to overestimation of the clinical impact of RIPC.

Another major limitation of the existing studies is the predominant focus on isolated clinical endpoints, without parallel assessment of the underlying biological responses. The absence of mechanistic endpoints has therefore hindered the interpretation of the inconsistent results of the trials conducted so far (Ahmed et al., 2019; Balin & Kivrak, 2019; Saes et al., 2013). Currently, there is a limited understanding of why RIPC may succeed or fail in IC.

Despite these limitations, RIPC remains an attractive intervention in IC due to its simplicity, non-invasive nature and low cost. Moreover, some of its proposed effects overlap conceptually with those of exercise therapy, which is known to be highly effective in IC, underscoring the importance of well-designed randomised trials to determine the effectiveness of RIPC in IC.

## 2.8. Summary of the literature review

LEAD is a common atherosclerotic disease affecting more than 230 million people worldwide (Fowkes et al., 2013; Song et al., 2019). IC is a common manifestation of LEAD and has a substantial impact on walking capacity, QoL and cardiovascular risk. Functional limitations in IC cannot be explained solely by impaired limb perfusion. A multifactorial pathophysiology, including skeletal muscle dysfunction, OS and metabolic derangements, contributes to exercise intolerance in IC (McDermott et al., 2020). SET is the first-line management of IC, but it has limited availability and is often poorly adhered to by patients (Mazzolai et al., 2024). Pharmacological options for symptom relief in IC are limited, and revascularisation is indicated only in the presence of lifestyle-limiting symptoms refractory to optimal medical therapy and SET (Nordanstig et al., 2024). Consequently, there is ongoing interest in developing adjunctive, non-invasive treatments that could meaningfully improve walking performance in IC.

RIPC is an experimental intervention in which brief episodes of non-injurious ischaemia and reperfusion in one vascular bed, such as the upper limb, activate endogenous protective pathways to protect distant ischaemia-sensitive tissues against more sustained ischaemia (Heusch et al., 2015). The concept of RIPC has been well established in animal models. A single bout of RIPC has been extensively studied in preventing organ damage related to cardiac and non-cardiac surgery, but the results have been inconsistent (Benstoem et al., 2017; Liang et al., 2023). In recent years, attention has shifted from a single episode of RIPC into repeated RIPC in chronic cardiovascular conditions, e.g. IC. Repeated RIPC may exert effects distinct from a single episode, including more sustained

vascular and microvascular adaptations, improved angiogenesis and modulation of gene expression (Lang & Kim, 2022). To date, only a limited number of trials have assessed the effects of repeated RIPC in IC. The results have been mixed and inconclusive, potentially due to heterogeneity in RIPC protocols, inadequate sham controls and limited confirmation of true ischaemia during intervention delivery. Furthermore, whether exogenous RIPC can confer additional benefit in a population that may already experience endogenous preconditioning through chronic ischaemia-reperfusion remains an open question, and is a central uncertainty motivating this investigation.

In addition to clinical outcomes, there is increasing interest in understanding the biological effects of RIPC through biomarkers of vascular function, oxidative stress and end-organ injury, as well as through metabolomic profiling. These approaches provide insight into the underlying mechanisms of RIPC and may help identify subtle systemic and tissue-level adaptations that are not captured by functional endpoints alone. Current evidence regarding the impact of repeated RIPC on these biological pathways in IC remains limited.

Therefore, there is a clear need for rigorously designed, sham-controlled randomised trials to evaluate the effects of RIPC in patients with IC. Combining clinical outcome measures with mechanistic endpoints is essential to determine the actual biological and functional impact of RIPC in IC, and to assess whether it influences not only symptoms but also vascular biology, cardiovascular risk and potentially long-term prognosis.

# 3. AIMS OF THE THESIS

The general aim of this thesis has been to evaluate whether repeated RIPC induced clinically meaningful improvements and measurable subclinical effects in patients with IC. This thesis has sought to assess the impact of daily RIPC on functional capacity of LEAD patients with IC and to explore vascular, biochemical and metabolic mechanisms underlying any observed effects.

Specific aims:

1. To determine whether repeated RIPC is a safe procedure with high adherence that improves symptoms, performance status and QoL in LEAD patients with IC (Paper I).
2. To assess the effects of repeated RIPC on arterial stiffness, and biomarkers related to inflammation, OS and end-organ damage in LEAD patients with IC (Paper II).
3. To characterise the skeletal muscle and systemic metabolic responses to RIPC with use of targeted metabolomic analyses and to identify the alterations at pathway-level that may enhance understanding of the effects of RIPC (Papers III–IV).

# 4. METHODS

## 4.1. Overview of the studies (Papers I–IV)

This PhD thesis is based on two single-centre, sham-controlled randomised clinical trials investigating the effects of RIPC in different clinical contexts. The main trial, which forms the core of this thesis (Papers I–III), evaluated the effects of repeated RIPC in LEAD patients with IC. The primary aim was to determine whether daily RIPC over 28 days improves walking performance (Paper I). In addition to clinical outcomes, the trial included prespecified mechanistic assessments, including arterial stiffness, biomarkers of OS and end-organ injury (Paper II), and targeted metabolomic profiling of serum and skeletal muscle (Paper III). These outcomes were designed to provide insight into the potential effects of RIPC and to aid in interpreting clinical findings. Paper V provides a theoretical perspective on the possible effects of RIPC in IC, complementing the empirical findings of Papers I–III.

In addition, the thesis incorporates findings from a separate single-centre, sham-controlled randomised trial conducted in patients undergoing elective vascular surgery (Paper IV). A metabolomic substudy of this trial evaluated the effects of a single preoperative RIPC episode on systemic metabolic responses in patients undergoing elective vascular surgery. The findings are intended to complement the results of the main trial with the metabolomic findings. Paper IV methods are described separately in Section 4.8, Overview of methods and statistical analyses for metabolites in the complementary vascular surgery RIPC study (Paper IV).

## 4.2. Study population and recruitment (Papers I–III)

### 4.2.1. Study population

Eligible participants were men aged 18–80 years with LEAD (ABI $\leq 0.9$ in the symptomatic leg) and typical IC (Fontaine stage IIa or IIb [Nordanstig et al., 2024]). The claudication distance was reported by the patient, and the diagnosis of IC had previously been established and classified by a vascular surgeon. The primary limitation of walking was claudication, not any other condition. Upon recruitment to the study, their disease was considered stable, and patients were on stable medical treatment. Changes in medication or revascularisation procedures within the past three months and during participation in the trial were avoided, and baseline visits were postponed for three months for patients with recent treatment changes. Before completing the study, no additional lifestyle advice was given to the patients by the trial investigators.

Patients with the following conditions were excluded: resting systolic BP > 200 mmHg, LEAD with Fontaine stage III or IV (ulcer or gangrene), severe renal insufficiency (estimated glomerular filtration rate < 30 mL/min/1.73 $m^2$), home based oxygen therapy or severe chronic obstructive pulmonary disease (Global

Initiative for Chronic Obstructive Lung Disease [GOLD 3–4]), severe heart failure (New York Heart Association [NYHA III–IV]), angina pectoris, hospitalisation for coronavirus disease 2019 during the past three months, history of malignancy (within the previous five years), persistent or permanent atrial fibrillation or atrial flutter, acute myocardial infarction in the past three months, residual signs of cerebral infarction impeding movement, symptomatic atherosclerosis of upper limb arteries, history of deep vein thrombosis of the upper limb, diabetic polyneuropathy and inability to give informed consent or to follow the study regimen.

### 4.2.2. Recruitment

From January 2022 to March 2023, patients in the vascular surgery outpatient clinic at Tartu University Hospital were invited to participate in the study. The researcher contacted consenting patients by phone to acquire oral informed consent. At the first visit to the research centre, patients were assessed for eligibility, and written informed consent was obtained before enrolment in the study.

## 4.3. Study design (Papers I–III)

This single-centre, parallel, randomised, sham-controlled, double-blind clinical trial took place from January 2022 to April 2023 in outpatient settings at Tartu University Hospital's Surgery Clinic in Estonia. Patients were allocated to receive RIPC or sham at a ratio of 1:1. Before starting the trial, it received ethical approval from the University of Tartu Ethics Committee (345/T-10) and was registered in the ClinicalTrials.gov database (NCT05084066). The trial was conducted in accordance with the Declaration of Helsinki and reported according to the Consolidated Standards of Reporting Trials (CONSORT) statement (Hopewell et al., 2025; Schulz et al., 2010).

## 4.4. Intervention and comparator (Papers I–III)

The RIPC protocol consisted of four cycles of 5 minutes of ischaemia followed by 5 minutes of reperfusion, and took a total of 35 minutes (Karbanda et al., 2009). RIPC was delivered by a patented automated remote ischaemic conditioning (RIC) device (autoRIC; StarFish Medical, Victoria, BC, Canada). The patient placed the autoRIC device on the upper non-dominant arm. After pressing the start button, the cuff inflated to 200 ± 5 mmHg for 5 minutes (inducing and ensuring ischaemia) and then deflated for 5 minutes (reperfusion) over a total of four cycles. The sham device was visually identical to the autoRIC device and generated the same sound and vibration effects, but the cuff was not actually inflated. At the first visit to the research centre, the device was introduced to the patients, who were taught to use it daily at home for the following 27 days, at approximately the same time of the day. The first procedure was carried out under the supervision of a research assistant.

## 4.5. Assessment of compliance and safety (Papers I–III)

The autoRIC applicator cuff mobile app (StarFish Medical, Victoria, BC, Canada) allowed compliance after the intervention to be evaluated, since it tracked and stored all complete RIPC procedures that were delivered with each individual cuff. To increase compliance, patients were supplied with a calendar to record the dates they used the device. They were free to call the research assistant whenever they had problems with the device. The research assistant called the patients weekly to remind them to use the device and to inquire about their health, any possible side effects and any problems with the device. At the end of the trial, participants completed a structured questionnaire designed to evaluate the usability of the device, any discomfort experienced during its use, and the occurrence of potential side effects.

## 4.6. Randomisation and blinding (Papers I–III)

### 4.6.1. Randomisation

The random allocation sequence was generated with the free computer software tool WINPEPI (PEPI-for-Windows) (Abramson, 2011). Varying block sizes of two and four were used, and patients were stratified according to their smoking status (active smoker or not) and age ($\geq 65$ years or $< 65$ years). Randomisation was prepared by a third party, and random allocation sequences were implemented using sequentially numbered opaque envelopes. The random allocation sequence and opaque envelopes were prepared by different people, who assigned the participants to interventions.

### 4.6.2. Blinding

This was a double-blind study, meaning that both the participants and researchers (who contacted the patients, made measurements and assessed outcomes) were blinded to group allocation. Adequate blinding of the participants was assumed because the sham device was externally identical to the RIPC device, but the cuff was not inflated during the episodes. After allocation to the study group, participants were only delivered the device that they were expected to use at home; hence, the participants did not know what the other device would look or feel like.

## 4.7. Sample size (Paper I)

Sample size calculation was performed based on MWD, the primary outcome measure. It was hypothesised that an improvement of 10% in MWD would be clinically significant. Based on a previous study (Balin & Kivrak, 2019), it was estimated that the average change in MWD would be 2% in the sham group and 20% in the RIPC group, with a standard deviation of 14%. To achieve 80% power

at a significance level of .050, 18 patients were needed for each group. Considering possible dropouts, it was planned to recruit 20 patients into each group.

## 4.8. Outcome measures (Papers I–III)

### 4.8.1. Walking distance and quality of life (Paper I)

The pre-defined primary outcome measure was the change in MWD after 28 days of RIPC compared with sham. The MWD was assessed at baseline and at the second visit using an incremental treadmill test (constant speed 3.2 km/hour, incline 0%, which increased 2% every 2 minutes, with a maximum of 8%). The pre-defined secondary outcomes were change in ICD, defined as change in the pain-free walking distance and change in time to relief from claudication (TRC), defined as the time (in seconds) during which the symptoms of claudication subsided. Another pre-defined secondary outcome measure was change in QoL, which was assessed with the Vascular Quality of Life Questionnaire-6 (VascuQoL-6) questionnaire (Nordanstig et al., 2014) at baseline and at the second visit. The questions were rated on a four-point scale, where one was the worst score, and four was the best score. The total scores varied from 6 to 24 points (Hageman et al., 2022; Nordanstig et al., 2014).

### 4.8.2. Arterial stiffness (Paper II)

The pre-specified secondary outcomes also included change in AIx, heart rate corrected AIx (AIx@75) and carotid-femoral PWV (cf-PWV) from baseline to 28 days post-intervention. These were measured using a cuff-based SphygmoCor XCEL 7.0 central BP and PWV measurement system (AtCor Medical Pty Ltd., Sydney, Australia). To ensure precision, all measurements were taken at least twice, and the median value was used. A third measurement was performed if the first two differed significantly: (1) aortic systolic BP > 10 mmHg, (2) AIx > 2 percentage points, (3) AIx@75 > 1 percentage point or (4) PWV > 0.5 m/s. The quality of measurements was assessed with the SphygmoCor XCEL internal quality control indicator, and only high-quality measurements were included in the analysis.

### 4.8.3. Biomarkers of inflammation, oxidative stress and end-organ damage (Paper II)

#### 4.8.3.1. Biomarkers of inflammation and oxidative stress (Paper II)

Inflammation and OS were assessed as a change in high-sensitivity C-reactive protein (hs-CRP), IL-6, IL-18, oxidised low-density lipoprotein (oxLDL), adiponectin and myeloperoxidase (MPO). Hs-CRP and IL-6 were measured from serum at the United Laboratories of the Tartu University Hospital according to a standard clinical laboratory protocol. The other markers were analysed from

serum at the Institute of Biomedicine and Translational Medicine, University of Tartu using the following kits: human total IL-18/IL-1F4 Quantikine enzyme-linked immunosorbent assay (ELISA) kit (Bio-Techne Ltd., Abingdon, UK), oxLDL ELISA kit (Mercodia, Uppsala, Sweden), human total adiponectin/Acrp20 Quantikine ELISA kit (Bio-Techne Ltd.) and human MPO Quantikine ELISA kit (Bio-Techne Ltd.).

Measurements of arterial stiffness and blood from the cubital vein were collected in the morning, both at baseline and 28 days post-intervention. Patients were advised not to eat or drink (except water), smoke for eight hours, or take medications on the morning of sample collection. Blood samples were centrifuged and the serum was separated and stored at -80 °C. All samples were analysed simultaneously after the end of the trial.

#### 4.8.3.2. Organ damage biomarkers (Paper II)

Myocardial injury was assessed as a change in high-sensitivity troponin T (hs-TnT) and N-terminal pro B-type natriuretic peptide (NT-proBNP). Kidney injury was assessed as a change in conventional kidney function biomarkers (creatinine, cystatin C and β2 microglobulin, neutrophil gelatinase associated lipocalin [NGAL] and kidney injury molecule 1 [KIM-1]).

Conventional heart and kidney function biomarkers were measured from serum at the United Laboratories of the Tartu University Hospital according to standard clinical laboratory protocols. NGAL and KIM-1 were analysed at the Institute of Biomedicine and Translational Medicine at the University of Tartu. NGAL was measured from serum using a human lipocalin-2/NGAL Quantikine ELISA kit (Bio-Techne Ltd., Abingdon, UK), and KIM-1 was measured from urine using a human urinary TIM-1/KIM-1/HAVCR ELISA kit (Bio-Techne Ltd.).

### 4.8.4. Metabolomic analyses on serum and skeletal muscle tissue (Paper III)

The prespecified exploratory outcomes were changes in low-molecular-weight metabolite levels in serum and muscle from baseline to 28 days, as compared between the RIPC and sham groups.

**Collection of serum and muscle biopsies**

Blood was collected from peripheral vein at baseline and at the 28-day follow-up visit, with follow-up sampling performed approximately 24 hours after the final RIPC session. The patients were advised not to eat, drink (except water) or smoke for eight hours, and not to take medications on the mornings of sample collection. Blood samples were centrifuged, and the serum was separated and stored at -80 °C. All samples were analysed simultaneously after the end of the trial.

Muscle biopsy from the symptomatic leg's gastrocnemius muscle was taken with a Precisa Tru-Cut 14 G, 10 cm needle (Mekalasi Oy, Helsinki, Finland)

under the guidance of ultrasound to ensure that the specimen was taken directly from the muscle. Five millilitres of 1% Lidocaine were used for local analgesia. A total of 1–3 attempts were made to obtain a muscle specimen of approximately 5–10 mg. After obtaining the specimen, it was immediately frozen in liquid nitrogen and stored at -80 °C until analysis. During baseline and follow-up visits, the muscle biopsies were obtained shortly after the treadmill test to detect ischaemic or acute post-ischaemic changes. All biopsy specimens were analysed simultaneously at the end of the trial.

**Muscle biopsy preparation for analysis**
Each specimen was weighed before analysis. The muscle tissue was homogenised as follows: 50 µL of phosphate-buffered saline and 10 homogenisation beads (SSB14B 1.4 mm Stainless Steel Beads 0.9–2.0 mm blend, [Next Advance, Inc, NY, USA]) were added to each microtube containing a muscle biopsy. Next, the specimens were homogenised twice for 5 minutes (Bullet Blender 24 Gold+ [Next Advance, Inc., NY, USA]), followed by 10 minutes at the Universal 32R centrifuge at a speed of 18840 relative centrifugal force. Consequently, 10 µL of supernatant from each tube were used for metabolomics measurements, and an additional 5 µL were used for protein concentration measurements. Protein concentration was measured with the Bio-Rad DC Protein assay kit (DC Protein Assay Kit, Bio-Rad Laboratories, Inc, CA, USA).

**Measurement of metabolites**
Metabolite profiling was performed using the Biocrates MxP® Quant 500 kit (Biocrates Life Sciences AG, Innsbruck, Austria) according to the manufacturer's protocol. Briefly, 10 µL of muscle specimen supernatants or serum were pipetted onto a 96-well plate containing internal standards, dried, derivatised with phenylisothiocynate, and re-extracted with ammonium acetate in methanol. The resulting extracts were split for analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for compounds requiring chromatographic separation, such as amino acids, biogenic amines and bile acids, and flow injection analysis-tandem mass spectrometry (FIA-MS/MS) for lipids and the other metabolites. Data processing and quality control were performed with the MetIDQ® software.

## 4.9. Statistical analyses (Papers I–IV)

All statistical analyses were conducted using the R software, version 4.3.1. (R Foundation for Statistical Computing, Vienna, Austria). The analysis followed a modified intention-to-treat approach; one participant in the sham group was excluded due to missing follow-up measurements. Data distribution was assessed using the Shapiro-Wilk test. Continuous baseline characteristics were presented as means with standard deviations for normally distributed variables and as medians with interquartile ranges (IQR) for non-normally distributed variables. Categorical variables were reported as counts and percentages. Between-group

comparisons were performed using the Student's *t*-test or Wilcoxon rank-sum test for continuous variables, and the $\chi^2$ test (for expected cell counts of five or more) or Fisher's exact test (for expected cell counts of less than five) for categorical variables.

### 4.9.1. Statistical analysis for primary outcome and other clinical outcomes (Paper I)

Two-way repeated measures analysis of variance (ANOVA) was performed to evaluate the main and interaction effects of intervention group and time regarding MWD, ICD and TRC. To better meet the assumptions of normality and homogeneity of variance for ANOVA, baseline and follow-up values of MWD, ICD and TRC were logarithmically transformed (natural logarithm), and analyses were performed on the transformed data. A $p$ value of $< .05$ was considered statistically significant.

### 4.9.2. Statistical analysis for biomarkers of inflammation, oxidative stress and end-organ damage (Papers II, V)

Data distribution was assessed using the Shapiro-Wilk test. Due to non-normal distributions, continuous variables were reported as medians with IQR. Comparisons between groups were done using the Wilcoxon rank sum test for continuous variables. The CI for differences in medians between groups was estimated using bootstrap resampling, implemented via the 'boot' package in R. This involved generating 1,000 bootstrap samples, with CIs derived using the percentile method based on the resampled distributions. The Bonferroni correction was applied to adjust the significance level for multiple testing, setting the threshold at .001 (.05 per 48 tests).

### 4.9.3. Overview of metabolomic analyses in acute and chronic ischaemic settings (Papers III–IV)

To evaluate whether RIPC induces measurable metabolic responses in humans, metabolomic analyses were conducted in two complementary settings. First, the effects of repeated RIPC on metabolic profiles were examined in patients with IC within the main randomised trial, using targeted metabolomic analyses of both serum and skeletal muscle samples (Paper III). Second, systemic metabolic effects of a single episode of RIPC were assessed in a randomised sham-controlled study of patients undergoing major vascular surgery (Paper IV), which represents an acute ischaemic stress model. Together, these analyses were intended to determine whether RIPC induces metabolic adaptations in a chronic ischaemic condition and to compare these with metabolic responses observed acutely under pronounced ischaemic stress.

### 4.9.4. Statistical analyses for metabolites of serum and skeletal muscle tissue (Paper III)

Metabolites with more than 30% missing values were excluded from the main analysis. Remaining missing metabolite values were imputed using the 'missForest' package of R (Stekhoven & Bühlmann, 2012). The metabolite concentrations measured in muscle tissue were normalised to the total protein content of each sample. To reduce right-skewness and to preserve relative concentration differences, the metabolite concentrations were log-transformed prior to downstream analyses.

To assess longitudinal changes in the log-transformed metabolite levels between the study groups, linear mixed-effects models were applied using the 'lme4' package of R. Models included fixed effects for group (sham vs. RIPC), time (baseline vs. follow-up) and their interaction, as well as a random intercept for participant to account for within-individual repeated measurements. The group × time interaction term was used to estimate differential changes in metabolite levels over time between sham and RIPC. Effect sizes from this interaction were interpreted as log fold changes and were converted to log2 fold changes for reporting and visualisation. Statistical significance was assessed using Wald *t*-tests with Satterthwaite approximation of degrees of freedom, as implemented in the 'lmerTest' package. Due to multiple testing *p* values were adjusted across metabolites using the Benjamini-Hochberg false discovery rate (FDR) procedure.

For multivariate analyses, log fold changes for each metabolite were calculated as the difference between log-transformed concentrations at follow-up and baseline. Metabolites with zero variance across samples were excluded prior to analysis. Principal component analysis was performed on the resulting matrix of log fold changes after mean-centering and scaling to unit variance using the prcomp() function of R. The proportion of variance explained by each principal component was calculated to aid interpretation. Associations between the first ten principal components and group assignment were assessed using linear regression models. Regression coefficients represent mean differences in principal components between sham and RIPC groups. The *p* values were adjusted for multiple testing using the Benjamini-Hochberg FDR procedure. An FDR < .05 were considered statistically significant.

### 4.9.5. Overview of methods and statistical analyses for metabolites in the complementary vascular surgery RIPC study (Paper IV)

Paper IV is based on an independent clinical trial conducted prior to the main study by the same investigators (Kasepalu, 2020), and only its metabolomics substudy is included in this thesis as complementary data to support and contextualise the metabolomic findings of the main study. The aim of the complementary vascular surgery RIPC study was to evaluate whether preoperatively performed RIPC affects the metabolome and to assess whether metabolomic changes

correlate with heart and kidney injury markers after vascular surgery. In brief, it was a single-centre, sham-controlled, double-blind trial (Kepler et al., 2019). Patients undergoing open abdominal aortic aneurysm repair, surgical lower limb revascularisation surgery (for IC or CLTI, common femoral artery endarterectomy, aorto(bi)femoral or femoropopliteal or femorotibial or iliofemoral bypass surgery) or carotid endarterectomy (for symptomatic or asymptomatic carotid stenosis) were recruited non-consecutively. The RIPC protocol, consisting of 4 cycles of 5 minutes of ischaemia, followed by 5 minutes of reperfusion, was applied. A single RIPC or a sham procedure was performed using a manual BP cuff on an upper arm, along with preparation for anaesthesia. Blood was collected preoperatively and 24 hours postoperatively. The serum metabolites were analysed using the AbsoluteIDQ p180 Kit (Biocrates Life Sciences AG, Innsbruck, Austria).

The RIPC and sham groups were compared using Student's *t*-test, and the Wilcoxon rank-sum or Chi-square test. Student's *t*-test was used in the case of a normal distribution and the Wilcoxon rank-sum test was applied in the case of a non-normal distribution. The Kolmogorov-Smirnov test was used to test for normality. Correlations were calculated using the Pearson or Spearman correlation coefficient. A $p$ value below .05 was considered significant for comparison of baseline characteristics. Because of multiple comparisons, the Benjamini-Hochberg procedure was used to control FDR. According to the Benjamini-Hochberg procedure, a new significance level was calculated, and a $p$ value below .0012 was considered significant for comparing changes in the metabolites and assessing correlations of the metabolites with heart and kidney injury markers. Metabolite enrichment analysis was performed with MetaboAnalyst 5.0 by using SMPDB and KEGG databases for which at least two intermediates were present.

# 5. RESULTS

## 5.1. Baseline characteristics of participants (Papers I–III)

Forty-two patients were enrolled and randomised to receive either RIPC or sham. Twenty-three patients from the RIPC and 18 from the sham group were included in the final analysis (Figure 1).

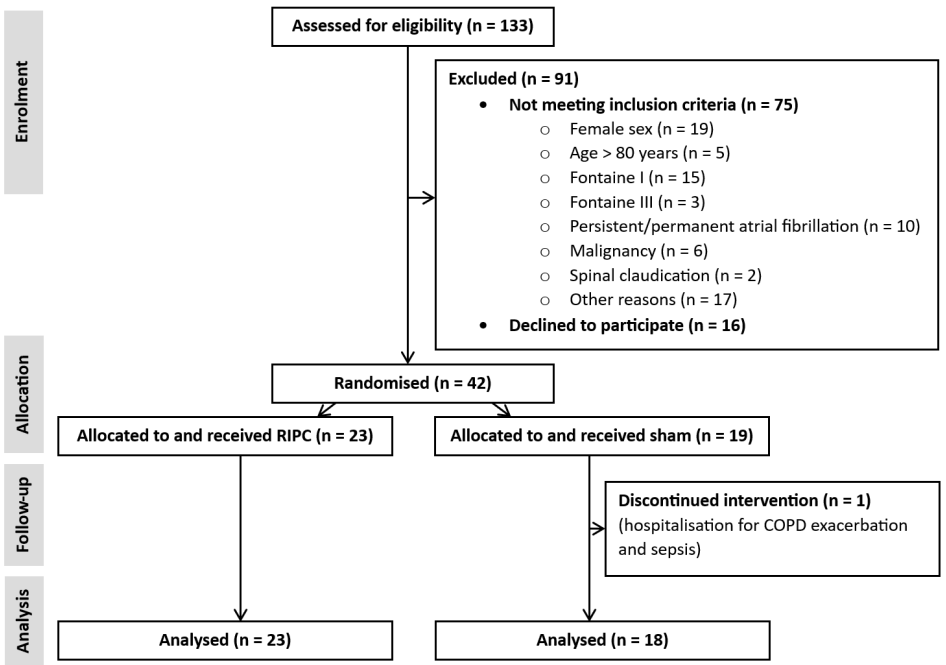


**Figure 1.** Consolidated Standards of Reporting Trials (CONSORT) flow diagram of a randomised, sham-controlled clinical trial evaluating repeated remote ischaemic preconditioning (RIPC) in patients with intermittent claudication. COPD = chronic obstructive pulmonary disease.

Baseline characteristics, including age, smoking status, severity and duration of LEAD, comorbidities and current medications were comparable between the RIPC and sham groups (Table 2).

**Table 2.** Baseline characteristics of participants ($n = 42$) in a randomised sham-controlled trial to assess the effects of repeated remote ischaemic preconditioning (RIPC) on intermittent claudication.

| ***Characteristic*** | ***Sham (n = 19)*** | ***RIPC (n = 23)*** | ***p value*** |
|---|---|---|---|
| Age (y) | 65.0 (61.0, 72.0) | 63.0 (62.5, 68.0) | .76 |
| Current smoker | 13 (68) | 16 (70) | .96 |
| Past smoker | 5 (26) | 6 (26) | |
| BMI (kg/m$^2$) | 28.0 (25.0, 31.9) | 27.7 (25.4, 33.3) | .73 |
| Fontaine IIa | 8 (42) | 11 (48) | .57 |
| Fontaine IIb | 11 (58) | 12 (52) | |
| Duration of LEAD (y) | 5.0 (2.0, 6.0) | 5.0 (3.0, 8.5) | .80 |
| ABI of right leg | 0.6 (0.5, 0.8) | 0.6 (0.5, 0.6) | .55 |
| ABI of left leg | 0.6 (0.5, 0.8) | 0.6 (0.5, 0.7) | .69 |
| Hypertension | 14 (74) | 18 (78) | .72 |
| Dyslipidaemia | 13 (68) | 19 (83) | .29 |
| Diabetes | 3 (16) | 7 (30) | .47 |
| Coronary artery disease | 3 (16) | 7 (30) | .47 |
| History of LEAD revascularisation | 5 (26) | 9 (39) | .25 |
| Heart failure | 2 (11) | 3 (13) | .80 |
| COPD | 3 (16) | 3 (13) | .80 |
| History of musculoskeletal disease | 7 (37) | 9 (39) | .99 |
| Antiplatelets | 11 (58) | 18 (78) | .12 |
| Antihypertensives | 13 (68) | 16 (70) | .84 |
| Beta blockers | 2 (11) | 7 (30) | .25 |
| Naftidrofuryl or pentoxifylline | 14 (74) | 19 (83) | .71 |
| Statins | 13 (68) | 15 (65) | .92 |
| Total cholesterol (mmol/l) | 4.6 (3.8, 5.3) | 4.1 (3.7, 5.8) | .87 |
| LDL cholesterol (mmol/l) | 2.7 (2.1, 3.6) | 2.5 (2.1, 4.0) | .80 |
| HDL cholesterol (mmol/l) | 1.1 (1.0, 1.4) | 1.1 (1.0, 1.3) | .65 |
| Triglycerides (mmol/l) | 1.4 (1.2, 1.6) | 1.6 (1.2, 2.0) | .45 |
| HbA1c (mmol/l) | 41 (38, 43) | 40 (36, 46) | .80 |
| HbA1c (%) | 5.9 (5.6, 6.1) | 5.8 (5.4, 6.4) | .80 |

*Data are presented as median (IQR) or n (%). Because of the small sample size, a non-normal distribution was assumed. Comparisons were made using the Wilcoxon rank sum test, $\chi^2$ test, or Fisher's exact test, as appropriate. ABI = ankle brachial index, BMI = body mass index; disease, COPD = chronic obstructive pulmonary disease, GOLD = Global Initiative for Chronic Obstructive Lung Disease, HbA1c = haemoglobin A1c, HDL = high density lipoprotein, LDL = low-density lipoprotein, LEAD = lower extremity arterial, NYHA = New York Heart Association, RIPC = remote ischaemic pre-conditioning.*

## 5.2. Effects of RIPC on walking distance and quality of life (Paper I)

**Comparison of claudication-related parameters between groups**

Baseline MWD, ICD and TRC were similar between the groups (Table 3). The MWD improved a median of 10.3% in the RIPC group compared with 6.9% in the sham group (between group median difference [MD] 6.8%, 95% CI –20.0 – 42.5; $p$ = .75). A pre-defined clinically significant change of > 10% in MWD occurred in 14 patients in the RIPC compared with eight patients in the sham group (relative risk 1.37, 95% CI 0.74 – 2.25; $p$ = .35). The ICD increased 34.7% in the RIPC group compared with 19.0% in the sham group (MD 15.4%, 95% CI –18.5 – 50.5; $p$ = .46). Claudication symptoms subsided 50 seconds faster in response to RIPC vs. 1 second in response to sham (MD –32 seconds, 95% CI –84 – 44; $p$ = .39) (Table 3). Individual changes in MWD and ICD are illustrated in Figure 2. There were no statistically significant effects of group or group × time interaction, but there was a statistically significant effect of time on improvement in MWD, ICD and TRC (Table 4).

**Table 3.** Comparison of baseline data and change in claudication in a randomised sham-controlled trial to assess the effects of repeated remote ischaemic preconditioning (RIPC) on intermittent claudication ($n$ =41).

| *Variable* | *Sham (n = 18)* | *RIPC (n = 23)* | *p value* |
|---|---|---|---|
| Maximal walking distance | | | |
| Baseline (m) | 160.7 (78.2, 420.8) | 181.3 (96.0, 285.3) | .74 |
| Follow-up (m) | 216.9 (90.5, 520.4) | 173.3 (97.5, 453.3) | .83 |
| Change (m) | 11.2 (–3.3, 59.3) | 24.3 (–6.2, 192.9) | .68 |
| Change (%) | 6.9 (–1.0, 38.1) | 10.3 (–4.3, 68.6) | .75 |
| Change by > 10 % | 8 (44) | 14 (61) | .30 |
| Intermittent claudication distance | | | |
| Baseline (m) | 75.6 (52.0, 118.0) | 83.6 (61.8, 125.8) | .60 |
| Follow-up (m) | 92.0 (65.5, 157.6) | 108.4 (64.4, 183.1) | .39 |
| Change (m) | 14.0 (2.0, 31.3) | 22.2 (3.0, 80.0) | .39 |
| Change (%) | 19.0 (2.4, 51.7) | 34.7 (2.4, 87.0) | .46 |
| Time to relief from claudication | | | |
| Baseline (s) | 153 (100, 223) | 164 (109, 205) | .95 |
| Follow-up (s) | 165 (140, 220) | 106 (84, 149) | .015 |
| Change (s) | –1 (–70, 51) | –50 (–83, 4) | .39 |

*Because of non-normal distribution, quantitative data are given as median (IQR) and compared using the Wilcoxon rank sum test. Binary data are given as n (%) and compared using the $\chi 2$ test.*

** Change in MWD was analysed as a continuous variable as well as a binary variable (improvement ≥ 10% vs. < 10%).*

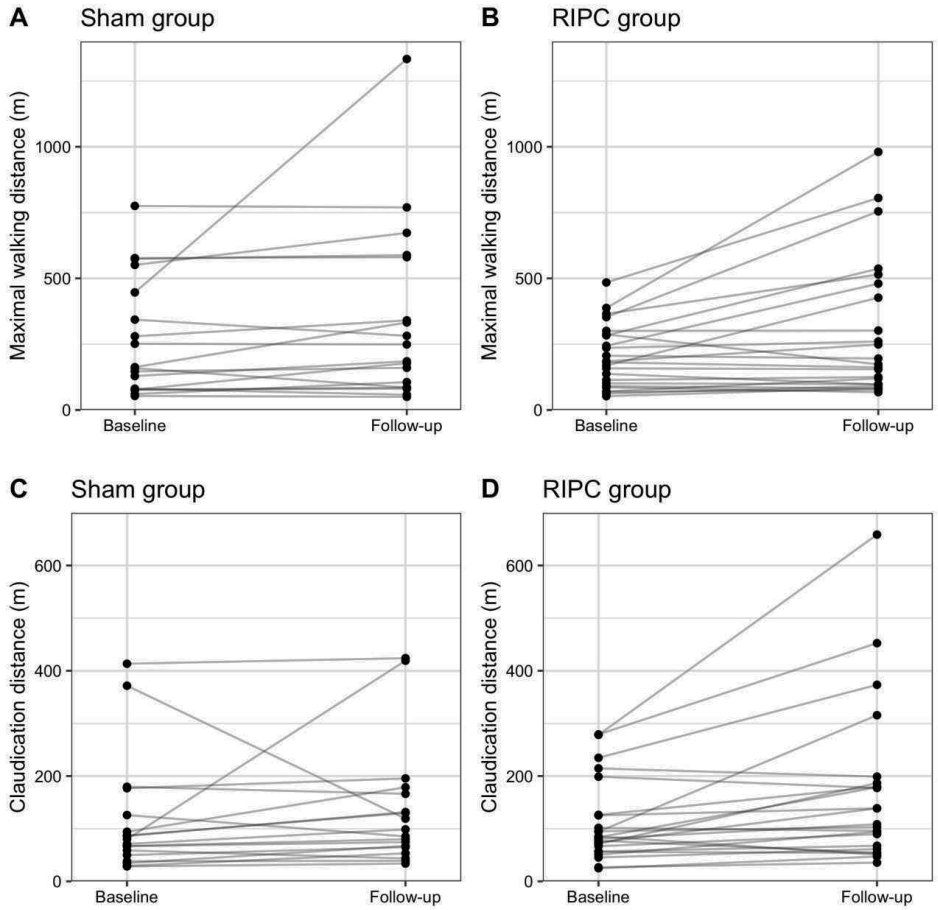


**Figure 2.** Individual changes in walking performance following 28 days of intervention in patients with intermittent claudication enrolled in a randomised, sham-controlled clinical trial of daily remote ischaemic preconditioning (RIPC). Panels (A) and (B) show changes in maximal walking distance, and panels (C) and (D) show changes in intermittent claudication distance. Panels (A, C) represent the sham group, and panels (B, D) represent the RIPC group. Measurements were obtained at baseline and after 28 days of daily intervention. Values are expressed in metres (m). Each line represents an individual patient.

**Table 4.** Comparison of claudication outcomes between the remote ischaemic preconditioning (RIPC) and sham groups in a randomised sham-controlled trial of daily RIPC in patients with intermittent claudication ($n$ = 41).

| *Outcome* | *Sham (n = 18)* | *RIPC (n = 23)* | *p values for two-way repeated measures ANOVA* | | |
|---|---|---|---|---|---|
| | | | *Group* | *Group x time interaction* | *Time* |
| Change in MWD (m) | 73.0 ± 211.9 | 96.2 ± 170.1 | .72 | .61 | .001 |
| Change in ICD (m) | 19.2 ± 105.3 | 57.1 ± 96.4 | .54 | .46 | .004 |
| Change in TRC (sec) | -68 ± 205 | -38 ± 71 | .17 | .16 | .015 |

*Data are given as mean ± standard deviation. ANOVA = analysis of variance, ICD = intermittent claudication distance, MWD = maximum walking distance, RIPC = remote ischaemic preconditioning, TRC = time to relief from claudication.*

*Original values of changes in MWD, ICD and TRC are presented. Natural logarithm was taken from the original values of MWD, ICD and TRC; logarithmic data were used in the analysis; corresponding p values are reported. Group, group × time interaction, and time effects were analysed with two way repeated measures ANOVA.*

## Health related quality of life

The mean baseline VascuQoL-6 score ± standard deviation was 14 ± 3 points in the RIPC group and 13 ± 4 points in the sham group ($p$ = .32), indicating similar baseline values between groups. The mean change in VascuQoL-6 score was 0 ± 2 points in the RIPC group vs. 0 ± 3 points in the sham group ($p$ = .84), demonstrating no difference between the groups.

## Compliance

Patients in both groups showed high compliance with the procedure in 95.8 ± 11.1% of the days in the RIPC and 99.6 ± 2.1% of the days in the sham group, without no statistically significant difference between groups ($p$ = .12). For logistic reasons, some participants used the device on one to two more days than initially planned (e.g., during the weekends).

Of the 41 patients, 37 (90%) rather or completely agreed that the device was easy to use, and 33 (80%) rather or completely agreed that it was easy to remember to use the device daily. Seven patients (17%) encountered frequent errors or obstacles with the device and had to contact the researcher by phone for help. Ten patients (56%) from the sham group and 11 patients (48%) from the RIPC group ($p$ = .96) rather or completely agreed that the device felt comfortable on the arm. No patient reported any side effects attributable to the use of the device during or after the trial.

## 5.3. Effects of RIPC on arterial stiffness, organ damage and oxidative stress (Papers II, V)

**Haemodynamics and arterial stiffness**

Baseline haemodynamic and arterial stiffness parameters were similar between the RIPC and sham groups (Table 5). After 28 days of intervention, peripheral diastolic BP decreased by a median of –2 mmHg (IQR –8, 1) in the RIPC group compared with no change in the sham group (median 0 mmHg, IQR –1, 2; $p$ = .040). The median difference between the groups was –2 mmHg (95% CI –8, 1). Central diastolic BP decreased by a median of –2 mmHg (IQR –7, 1) in the RIPC group compared with an increase of 1 mmHg (IQR –1, 3) in the sham group ($p$ = .033). The median difference between the groups was –2 mmHg (95% CI –8, 0). However, after correction for multiple comparisons, these changes were not statistically significant. Changes in the other parameters of arterial stiffness also remained statistically insignificant between the groups (Table 5).

**Biomarkers for end-organ damage, inflammation and oxidative stress**

The biomarkers for baseline cardiac and renal function as well as OS were similar between groups (Table 6). In response to 28 days of intervention, NGAL, a marker of renal injury, increased by a median of 3 ng/mL (IQR –9, 16) in the RIPC group compared with a decrease of –9 ng/mL (IQR –14, 5) in the sham group ($p$ = .044). The median difference between the groups was 11 ng/mL (95% CI –9, 27); however, after correcting for multiple testing, the change was not statistically significant. No other nominally significant changes were observed in the biomarkers for end-organ damage or OS following repeated RIPC (Table 6).

**Table 5.** Baseline haemodynamic and arterial stiffness parameters and their change from baseline for participants ($n$ = 41) in a randomised trial to assess the effects of remote ischaemic preconditioning (RIPC) on arterial stiffness, inflammation, oxidative stress and organ damage in patients with intermittent claudication.

| ***Characteristic*** | ***Baseline*** | | | ***Change*** | | |
|---|---|---|---|---|---|---|
| | ***Sham (n = 18)*** | ***RIPC (n = 23)*** | ***p value*** | ***Sham (n = 18)*** | ***RIPC (n = 23)*** | ***p value*** |
| Heart rate (bpm) | 61 (57, 76) | 69 (61, 77) | .47 | 0 (–3, 9) | –1 (–3, 3) | .41 |
| Peripheral SBP (mmHg) | 152 (136, 158) | 149 (137, 156) | .73 | –2 (–9, 3) | –5 (–13, 11) | .78 |
| Peripheral DBP (mmHg) | 85 (75, 89) | 89 (76, 93) | .27 | 0 (–1, 2) | –2 (–8, 1) | .040 |
| Central SBP (mmHg) | 139 (128, 142) | 133 (124, 140) | .54 | –2 (–9, 10) | –1 (–10, 3) | .81 |
| Central DBP (mmHg) | 86 (77, 90) | 89 (77, 95) | .33 | 1 (–1, 3) | –2 (–7, 1) | .033 |
| Pulse pressure (mmHg) | 50 (39, 59) | 46 (41, 51) | .32 | –3 (–4, 1) | –1 (–3, 6) | .15 |
| MAP (mmHg) | 104 (97, 109) | 107 (95, 112) | .72 | 0 (–4, 2) | –3 (–7, 4) | .44 |
| AIx (%) | 31 (23, 36) | 26 (17, 33) | .14 | 2 (–2, 5) | 2 (–2, 6) | .98 |
| AIx@75 (%) | 25 (19, 36) | 23 (11, 31) | .36 | 2 (0, 8) | 3 (–1, 7) | .95 |
| cf-PWV (m/s) | 8.8 (7.7, 10.1) | 9.4 (8.3, 10.3) | .26 | 0.2 (–0.3, 0.8) | 0.2 (–0.6, 0.6) | .54 |

*Data are presented as median (IQR). Because of the small sample size, a non-normal distribution was assumed, and groups were compared using the Wilcoxon rank sum test. AIx = augmentation index, AIx@75 = heart rate corrected augmentation index, bpm = beats per minute, cf-PWV = carotid–femoral pulse wave velocity, DBP = diastolic blood pressure, RIPC = remote ischaemic preconditioning, SBP = systolic blood pressure.*

**Table 6.** Baseline biomarkers for inflammation, oxidative stress and end-organ damage and their change from baseline for participants ($n$ = 41) in a randomised sham-controlled trial to assess the effects of repeated remote ischaemic preconditioning (RIPC) on arterial stiffness, oxidative stress and organ damage in patients with intermittent claudication.

| ***Biomarker*** | ***Baseline*** | | | ***Change*** | | |
|---|---|---|---|---|---|---|
| | ***Sham (n = 18)*** | ***RIPC (n = 23)*** | ***p value*** | ***Sham (n = 18)*** | ***RIPC (n = 23)*** | ***p value*** |
| Hs-TnT (ng/L) | 10 (7, 14) | 11 (7, 17) | .99 | 1 (–1, 2) | 0 (–1, 2) | .54 |
| NT-proBNP (pg/mL) | 69 (53, 154) | 95 (56, 195) | .61 | 6 (–14, 35) | –7 (–32, 18) | .14 |
| Creatinine (μmol/L) | 84 (66, 95) | 85 (73, 101) | .36 | –1 (–9, 4) | –1 (–3, 6) | .34 |
| Urea (mmol/L) | 5 (4, 7) | 6 (4, 7) | .85 | 0 (–1, 1) | 0 (–0, 1) | .12 |
| Cystatin C (mg/L) | 0.9 (0.9, 1.2) | 1.0 (1.0, 1.3) | .34 | 0.0 (–0.1, 0.0) | 0.0 (–0.1, 0.0) | .45 |
| β2 microglobulin (μg/L) | 2165 (1928, 2658) | 2240 (2060, 2715) | .40 | –37 (–155, 220) | –10 (–136, 95) | .93 |
| NGAL (ng/mL) | 98 (85, 115) | 104 (91, 120) | .49 | –9 (–14, 5) | 3 (–9, 16) | .044 |
| Urinary KIM-1 (ng/mL) | 2 (1, 4) | 2 (2, 4) | .89 | 0 (–1, 1) | 0 (–1, 0) | .41 |
| Hs-CRP (mg/L) | 3 (1, 5) | 2 (1, 4) | .49 | 0 (–1, 1) | 0 (–1, 1) | .94 |
| IL-6 (ng/L) | 3 (2, 6) | 3 (2, 5) | .99 | 1 (–1, 2) | 1 (–1, 3) | .89 |
| IL-18 (pg/mL) | 213 (171, 273) | 247 (209, 327) | .16 | 5 (–13, 17) | 1 (–18, 15) | .89 |
| OxLDL (U/L) | 65 (58, 77) | 75 (60, 101) | .34 | 0 (–6, 11) | 0 (–11, 10) | .65 |
| MPO (ng/mL) | 445 (390, 587) | 593 (395, 752) | .25 | –80 (–165, 104) | 53 (–97, 78) | .47 |
| Adiponectin (ng/mL) | 3832 (3056, 5196) | 3436 (2962, 4670) | .58 | 131 (–269, 365) | –245 (–903, 270) | .32 |

*Data are presented as median (interquartile range). Because of the small sample size, a non-normal distribution was assumed, and comparisons were made using the Wilcoxon rank sum test. Hs-CRP = high-sensitivity C-reactive protein, hs-TnT = high-sensitivity troponin T, IL = interleukin, KIM-1 = kidney injury molecule 1, MPO = myeloperoxidase, NGAL = neutrophil gelatinase-associated lipocalin, NT-proBNP = N-terminal pro B-type natriuretic peptide, oxLDL = oxidised low-density lipoprotein, RIPC = remote ischaemic preconditioning.*

## 5.4. Effects of RIPC on metabolic profiles of serum and skeletal muscle (Papers III–IV)

### 5.4.1. Changes in the serum and skeletal muscle metabolites following repeated RIPC for 28 days (Paper III)

In total, 41 patients were included in the final analysis for serum metabolites: 23 patients from the RIPC group and 18 from the sham group. Furthermore, muscle metabolites from 16 patients (8 from each group) were analysed. Both the median time from the end of the treadmill test to the first muscle biopsy, as well as the median time from the end of claudication symptoms to the first muscle biopsy were similar between the groups (Table 7). No complications were associated with the muscle biopsy procedure.

**Table 7.** Comparison of muscle biopsy related characteristics between the remote ischaemic preconditioning (RIPC) and sham groups in a randomised sham-controlled trial to assess the effects of repeated RIPC on metabolome in patients with intermittent claudication.

| *Characteristic* | *RIPC (n = 23)* | *Sham (n = 18)* | *p value* |
|---|---|---|---|
| Participants with biopsy (baseline) | 12 (52) | 11 (61) | .95 |
| Participants with biopsy (follow-up) | 8 (35) | 8 (44) | .87 |
| Biopsies per participant (baseline) | 1.0 (1.0, 2.0) | 2.0 (1.0, 2.5) | .14 |
| Biopsies per participant (follow-up) | 2.0 (1.0, 2.3) | 2.0 (1.0, 2.0) | .84 |
| Treadmill end-to-biopsy time (baseline) (s) | 771 (698, 897) | 880 (625, 1168) | .61 |
| Treadmill end-to-biopsy time (follow-up) (s) | 725 (674, 778) | 663 (612, 990) | .65 |
| Claudication end-to-biopsy time (baseline) (s) | 624 (544, 718) | 580 (475, 828) | .83 |
| Claudication end-to-biopsy time (follow-up) (s) | 602 (536, 663) | 488 (470, 797) | .37 |

*Data are given as median (interquartile range) or count (percentage). Comparisons were made using the $\chi^2$ test or Wilcoxon rank-sum test, as appropriate. RIPC = remote ischaemic preconditioning, s = second.*

**Serum and muscle metabolites**

A total of 616 metabolites from 26 biochemical classes and 261 metabolic sums and ratios were assessed from serum samples. Fourteen metabolites were excluded because >30% of their baseline or follow-up values were below the level of detection (<LOD). No significant between-group differences were observed at baseline. No significant group × time interactions were observed for serum metabolites after 28 days of intervention (FDR > .05 for all metabolites; Figure 3A and Table 8), indicating that repeated RIPC over 28 days was not associated with statistically detectable changes in the serum metabolome. From muscle biopsy samples, 599 metabolites and 228 metabolic sums and ratios were analysed. Thirty-one metabolites were excluded because >30% of their baseline or follow-up values were < LOD. At baseline, none of the metabolites differed significantly between the groups. No statistically significant group × time interactions were

identified, FDR > .05 for all metabolites, suggesting no detectable effect of repeated RIPC on the muscle metabolome (Figure 3B and Table 9).

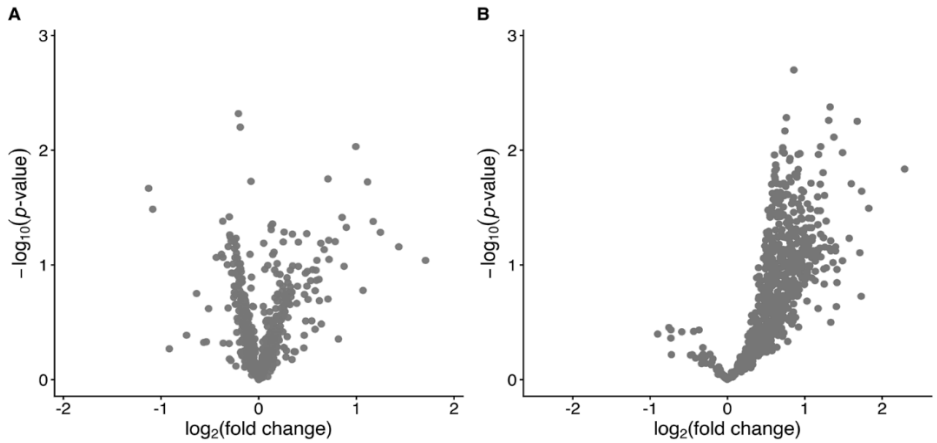


**Figure 3.** Volcano plots of serum (A) and skeletal muscle (B) metabolites showing group × time interaction effects comparing repeated remote ischaemic preconditioning with sham over 28 days. Each point represents one metabolite, plotted according to the estimated log2 fold change and −log10 of the nominal *p* value obtained from linear mixed-effects models. No metabolites showed statistically significant differential changes between groups after correction for multiple testing using the Benjamini-Hochberg false discovery rate.

**Table 8.** Comparison of changes in metabolite levels after 28 days of intervention between the remote ischaemic preconditioning (RIPC) and sham groups in serum (nominal $p < .05$) in a randomised trial to assess the effects of repeated RIPC on metabolome in patients with intermittent claudication.

| *Metabolite* | *Log2 fold change (group × time)* | *p value* | *FDR* |
|---|---|---|---|
| C3.DC.C4.OH. | −0.368 | .042 | .999 |
| Nitro.Tyr | −1.127 | .022 | .999 |
| GDCA | 0.897 | .047 | .999 |
| TDCA | 1.172 | .042 | .999 |
| Dopamine | −0.079 | .019 | .999 |
| Spermidine | −0.301 | .038 | .999 |
| Cer.d18.0.18.0.OH | 0.709 | .018 | .999 |
| Tyr.nitration | −1.085 | .033 | .999 |
| CDCA.CA | 1.114 | .019 | .999 |
| Sum.conjugated.secondary.BA | 0.994 | .009 | .999 |
| Sum.secondary.BA | 0.854 | .039 | .999 |
| PUFA.LPC.MUFA.LPC | 0.141 | .044 | .999 |
| MUFA.PC.SFA.PC | −0.189 | .006 | .999 |
| MUFA.aa.PC.SFA.aa.PC | −0.208 | .005 | .999 |

| *Metabolite* | *Log2 fold change (group × time)* | *p value* | *FDR* |
|---|---|---|---|
| PUFA.PC.MUFA.PC | 0.126 | .046 | .999 |
| PUFA.aa.PC.MUFA.aa.PC | 0.128 | .049 | .999 |

*Analyses were performed on log-transformed metabolite concentrations using linear mixed-effects models. Statistical significance was assessed using Wald t-tests. The p values were adjusted for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) procedure, with FDR <.05 considered statistically significant. aa = diacyl, BA = bile acid, C18 = stearoylcarnitine, C3 = propionylcarnitine, C4 = four-carbon acylcarnitine, CA = cholic acid, CDCA = chenodeoxycholic acid, Cer = ceramide, d = dihydroxy, DC = dicarboxylic, GDCA = glycodeoxycholic acid, LPC = lysophosphatidylcholine, MUFA = monounsaturated fatty acid, Nitro.Tyr = nitrotyrosine, OH = hydroxy, PC = phosphatidylcholine, PUFA = polyunsaturated fatty acid, SFA = saturated fatty acid, TDCA = taurodeoxycholic acid, Tyr = tyrosine.*

**Table 9.** Comparison of changes in metabolite levels after 28 days of intervention between the remote ischaemic preconditioning (RIPC) and sham groups in skeletal muscle (nominal $p < .05$) to assess the effects of repeated RIPC on metabolome in patients with intermittent claudication.

| *Metabolite* | *Log2 fold change (group × time)* | *p value* | *FDR* | *Metabolite* | *Log2 fold change (group × time)* | *p value* | *FDR* |
|---|---|---|---|---|---|---|---|
| C3.1 | 1.255 | .025 | .337 | Hex3Cer.d18.1.22.0 | 1.080 | .019 | .337 |
| C4.1 | 0.699 | .036 | .337 | Hex3Cer.d18.1.26.1 | 1.678 | .006 | .337 |
| C5.1 | 0.790 | .040 | .337 | Hex.Cer.d18.1.14.0 | 1.376 | .008 | .337 |
| C10 | 0.885 | .048 | .337 | Hex.Cer.d18.1.22.0 | 1.601 | .020 | .337 |
| C10.1 | 0.801 | .017 | .337 | Hex.Cer.d18.1.26.1 | 1.205 | .009 | .337 |
| C10.2 | 0.760 | .024 | .337 | Hex.Cer.d18.2.23.0 | 1.827 | .032 | .337 |
| C12.DC | 0.632 | .015 | .337 | TG.14.0_35.1 | 0.726 | .037 | .337 |
| C12.1 | 0.705 | .025 | .337 | TG.17.0_36.4 | 0.734 | .011 | .337 |
| C14.1.OH | 0.957 | .034 | .337 | TG.20.1_32.3 | 0.644 | .025 | .337 |
| C14.2.OH | 0.913 | .011 | .337 | TG.20.4_33.2 | 1.071 | .033 | .337 |
| C16.2.OH | 0.644 | .038 | .337 | TG.22.2_32.4 | 1.327 | .004 | .337 |
| Pro | 1.087 | .039 | .337 | Asn.synthesis | 0.608 | .011 | .337 |
| Tyr | 1.088 | .047 | .337 | Glutaminolysis | 0.753 | .021 | .337 |
| ADMA | 0.856 | .035 | .337 | PKU.NBS | 0.965 | .040 | .337 |
| Anserine | 1.276 | .042 | .337 | Nonessential.EssentialAA | 0.831 | .029 | .337 |
| Cit | 0.943 | .033 | .337 | Met1His.synthesis | 1.236 | .016 | .337 |
| Creatinine | 1.059 | .047 | .337 | AABA.synthesis | 0.812 | .047 | .337 |
| Met.SO | 0.743 | .007 | .337 | Muscle.degradation | 0.815 | .042 | .337 |
| SDMA | 1.010 | .048 | .337 | Sum.DMArg | 0.916 | .029 | .337 |
| GABA | 1.200 | .034 | .337 | SDMA.Arg | 0.715 | .010 | .337 |
| Serotonin | 0.639 | .023 | .337 | Sum.neurotransmitters | 0.675 | .038 | .337 |
| AconAcid | 0.790 | .046 | .337 | LDH.activity | 0.900 | .016 | .337 |
| OH.GlutAcid | 0.958 | .022 | .337 | Sum.FA | 0.551 | .038 | .337 |
| Cer.d18.1.18.0.OH | 1.488 | .011 | .337 | Sum.saturated.FA | 0.551 | .037 | .337 |
| Cer.d18.1.18.1 | 0.705 | .049 | .337 | Sum.n3.FA | 0.615 | .017 | .337 |
| Cer.d18.2.16.0 | 1.309 | .006 | .337 | MUFA.LPC.SFA.LPC | 0.858 | .002 | .337 |
| CE.15.1 | 1.048 | .039 | .337 | UFA.LPC.SFA.LPC | 0.687 | .038 | .337 |

| *Metabolite* | *Log2 fold change (group × time)* | *p value* | *FDR* | *Metabolite* | *Log2 fold change (group × time)* | *p value* | *FDR* |
|---|---|---|---|---|---|---|---|
| CE.16.1 | 1.735 | .023 | .337 | BKT.NBS | 0.722 | .031 | .337 |
| DG.14.0_14.0 | 0.820 | .019 | .337 | EMA.NBS | 1.167 | .045 | .337 |
| DG.14.1_18.1 | 0.845 | .027 | .337 | IBD.NBS | 0.955 | .026 | .337 |
| DG.16.0_16.0 | 0.580 | .035 | .337 | IVA.NBS | 0.894 | .042 | .337 |
| DG.16.0_16.1 | 0.939 | .032 | .337 | MA.NBS | 0.762 | .005 | .337 |
| DG.18.1_20.0 | 0.524 | .033 | .337 | MMA.NBS | 0.662 | .027 | .337 |
| DG.21.0_22.6 | 0.762 | .043 | .337 | PA.NBS | 0.826 | .049 | .337 |
| FA.16.0 | 0.615 | .036 | .337 | Sum.MC.carnitine | 0.856 | .026 | .337 |
| FA.18.0 | 0.663 | .028 | .337 | MC.LC.carnitine | 0.685 | .020 | .337 |
| LPC.14.0 | 0.732 | .025 | .337 | Sum.saturated.DG | 0.610 | .036 | .337 |
| LPC.16.1 | 1.177 | .011 | .337 | Sum.MUFA.CE | 1.164 | .034 | .337 |
| PC.24.0 | 0.666 | .044 | .337 | Sum.Cer1 | 0.721 | .036 | .337 |
| PC.26.0 | 0.711 | .010 | .337 | Sum.LCFA.Glycosyl.Cer | 0.788 | .039 | .337 |
| PC.28.1 | 0.897 | .017 | .337 | Sum.HexCer | 0.915 | .015 | .337 |
| PC.32.0 | 0.950 | .039 | .337 | Sum.Glycosyl.Cer | 0.873 | .016 | .337 |
| PC.40.1 | 0.618 | .018 | .337 | Sum.Hex3Cer | 0.727 | .019 | .337 |
| PC.42.2 | 0.623 | .013 | .337 | Cer.DHCer | 1.166 | .026 | .337 |
| PC.42.4 | 1.200 | .020 | .337 | GlcCer.Cer | 0.809 | .012 | .337 |
| PC.42.6 | 0.636 | .020 | .337 | Hex3Cer.Cer | 0.621 | .029 | .337 |
| PC.O.30.0 | 0.681 | .027 | .337 | HexCer.Cer | 0.804 | .012 | .337 |
| PC.O.30.1 | 2.291 | .015 | .337 | Sum.LCFA.Cer | 0.781 | .024 | .337 |
| PC.O.32.1 | 1.034 | .029 | .337 | Sum.LCFA.HexCer | 0.771 | .042 | .337 |
| PC.O.40.4 | 0.664 | .029 | .337 | Sum.VLCFA.Glycosyl.Cer | 0.937 | .011 | .337 |
| PC.O.42.0 | 0.581 | .024 | .337 | OCFA.SM.ECFA.SM | 0.638 | .028 | .337 |
| PC.O.42.1 | 0.573 | .032 | .337 | Sum.saturated.ae.PC | 0.685 | .023 | .337 |
| PC.O.42.5 | 0.606 | .017 | .337 | Sum.SFA.ae.PC | 0.685 | .023 | .337 |
| PC.O.44.4 | 0.614 | .015 | .337 | PCae.PCaa | 0.592 | .027 | .337 |
| PC.O.44.6 | 0.572 | .020 | .337 | PUFA.aa.PC.MUFA.aa.PC | 0.658 | .034 | .337 |
| Hex3Cer.d18.1.20.0 | 1.17 | .045 | .337 | PLA2.activity1 | 0.983 | .023 | .337 |

*Analyses were performed on log-transformed metabolite concentrations using linear mixed-effects models. Statistical significance was assessed using Wald t-tests. The p values were adjusted for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) procedure, with FDR <.05 considered statistically significant. AA = amino acid, AABA = alpha-aminobutyric acid, AconAcid = aconitic acid, ADMA = asymmetric dimethylarginine, ae = acyl-alkyl, Arg = arginine, Asn = asparagine, BKT = β-keto-thiolase deficiency, C = carnitine, CE = cholesteryl ester, Cer = ceramide, Cit = citrul-line, d = dihydroxy, DC = dicarboxylate, DG = diacylglycerol, DHCer = dihydrocer-amide, DMArg = dimethylarginine, ECFA = even-chain fatty acid, EMA = ethylmalonic acid, FA = fatty acid, GABA = gamma-aminobutyric acid, GlutAcid = glutamic acid, HexCer = hexosylceramide, His = histidine, IBD = isobutyric acid, IVA = isovaleric acidemia marker, LC = long chain, LDH = lactate dehydrogenase, LPC = lysophosphati-dylcholine, MA = methylmalonic acidemia marker, MC = medium chain, Met = methio-nine, Met.SO = methionine sulfoxide, MMA = methylmalonic acid(emia), MUFA = mono-unsaturated fatty acid, NBS = newborn screening, OCFA = odd-chain fatty acid, OH = hydroxy, OH = hydroxy, PA = propionic acidemia, PC = phosphatidylcholine, PKU = phenylketonuria, PLA2 = phospholipase A2, Pro = proline, SDMA = symmetric dimethylarginine, SFA = saturated fatty acid, TG = triglyceride, Tyr = tyrosine, UFA = unsaturated fatty acid, VLCFA = very-long-chain fatty acid.*

**Principal component analysis**

Principal component analysis of serum and muscle metabolites did not demonstrate a statistically significant association between any of the first 10 principal components and study groups (RIPC vs. sham), indicating similar global metabolic profiles between the groups (Table 10).

**Table 10.** The top 10 principal components compared between the remote ischaemic preconditioning (RIPC) and sham groups in serum and skeletal muscle to assess the effects of repeated RIPC on metabolome in patients with intermittent claudication.

| | *Serum* | | | | | *Skeletal muscle* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| ***PC*** | ***PC explained variance (%)*** | ***Beta*** | ***SE*** | ***p value*** | ***FDR*** | ***PC explained variance (%)*** | ***Beta*** | ***SE*** | ***p value*** | ***FDR*** |
| PC1 | 37.7 | −2.15 | 5.78 | .71 | .830 | 48.7 | 17.02 | 9.34 | .090 | .898 |
| PC2 | 8.7 | 2.68 | 2.76 | .34 | .511 | 15.7 | 4.93 | 5.76 | .41 | .907 |
| PC3 | 5.8 | −2.61 | 2.23 | .25 | .511 | 8.0 | −4.68 | 4.02 | .26 | .907 |
| PC4 | 4.3 | −2.55 | 1.91 | .19 | .511 | 4.3 | 0 | 3.10 | .99 | .999 |
| PC5 | 3.2 | −3.35 | 1.61 | .044 | .440 | 3.4 | −1.12 | 2.74 | .69 | .907 |
| PC6 | 3.1 | 1.53 | 1.64 | .36 | .511 | 2.8 | 0.05 | 2.51 | .99 | .999 |
| PC7 | 2.6 | −1.59 | 1.50 | .29 | .511 | 2.8 | 0.88 | 2.47 | .73 | .907 |
| PC8 | 2.3 | −1.77 | 1.40 | .22 | .511 | 2.6 | 1.07 | 2.39 | .66 | .907 |
| PC9 | 2.2 | 0.05 | 1.40 | .97 | .971 | 2.2 | 1.05 | 2.18 | .64 | .907 |
| PC10 | 2.1 | −0.44 | 1.37 | .75 | .830 | 2.0 | −2.09 | 2.03 | .32 | .907 |

*Analyses were performed on log-transformed data. Principal component (PC) analysis was performed on the log fold changes of metabolites. The proportion of variance explained by each PC was calculated to aid interpretation. Associations between the first 10 principal components and group assignment were assessed using linear regression models. Regression coefficients represent mean differences in principal components between sham and RIPC groups. The p values were adjusted for multiple testing using the Benjamini-Hochberg false discovery procedure (FDR). FDR < .05 was considered statistically significant. SE = standard error.*

### 5.4.2. Changes in the serum metabolites 24 h postoperatively following a single episode of RIPC in vascular surgery (Paper IV)

A total of 98 patients undergoing elective vascular surgery were enrolled and randomised into study groups. Final analysis included 45 patients from the RIPC group and 47 patients from the sham group (Figure 4). The median time from the end of the intervention to the beginning of surgery did not differ significantly ($p$ = .057) between the RIPC (36 min, IQR 21–46 min) and the sham group (25 min, IQR 15–38 min). The baseline characteristics of patients did not differ significantly between the study groups (Table 11).

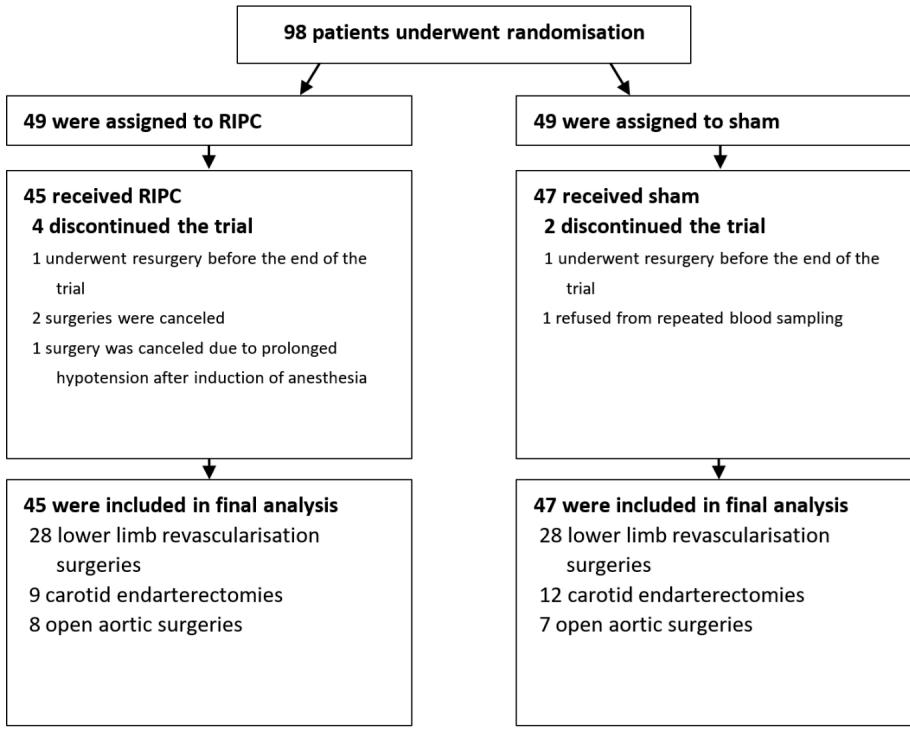


**Figure 4.** Patients' flow chart of a randomised trial to assess the effects of a single episode of remote ischaemic preconditioning (RIPC) on metabolome in patients undergoing vascular surgery.

**Table 11.** Baseline characteristics of participants ($n$ = 92) in a randomised trial to assess the effects of a single episode of remote ischaemic preconditioning (RIPC) on metabolome in patients undergoing vascular surgery.

| *Variable* | *Sham (n = 47)* | *RIPC (n = 45)* | *p value* |
|---|---|---|---|
| Age (years) | 66 (± 10) | 67 (± 9) | .57 |
| Male | 32 (68) | 36 (80) | .29 |
| BMI (kg/m$^2$) | 26.5 (± 6.7) | 26.3 (± 6.4) | .84 |
| ASA 2 | 19 (40) | 18 (40) | .99 |
| ASA 3 | 22 (47) | 20 (44) | .99 |
| ASA 4 | 6 (13) | 7 (16) | .99 |
| ACEI or ARB | 30 (64) | 21 (47) | .15 |
| Calcium channel blockers | 17 (37) | 9 (20) | .14 |
| Beta-blockers | 19 (40) | 11 (24) | .16 |
| Statins | 14 (30) | 13 (29) | .99 |
| Diabetes | 8 (17) | 5 (11) | .61 |
| Myocardial infarction | 3 (6) | 8 (18) | .17 |
| Stroke | 12 (26) | 10 (22) | .90 |
| Smoker (current or ex-smoker) | 42 (89) | 40 (89) | .99 |
| MAP (mmHg) | 100 (± 11) | 99 (± 12) | .68 |

| *Variable* | *Sham (n = 47)* | *RIPC (n = 45)* | *p value* |
|---|---|---|---|
| Heart rate (bpm) | 67 (± 11) | 66 (± 9) | .75 |
| Cholesterol (mmol/L) | 5.0 (3.9, 5.6) | 5.0 (4.2, 5.7) | .79 |
| LDL (mmol/L) | 3.3 (2.5, 3.8) | 3.4 (2.6, 3.9) | .50 |
| HDL (mmol/L) | 1.1 (1.0, 1.3) | 1.1 (0.9, 1.4) | .31 |
| Triglycerides (mmol/L) | 1.5 (1.2, 2.0) | 1.6 (1.3, 1.8) | .79 |
| Administration of propofol | 26 (55) | 19 (42) | .30 |
| Duration of surgery (min) | 112 (84, 156) | 108 (89, 135) | .83 |

*All p values were calculated for data with a normal distribution (presented as mean and standard deviation) using Student's t-test; for data with a non-normal distribution (presented as median and interquartile range) using the Wilcoxon rank-sum test; and for binary data (presented as number and percentage), using the χ2 test. ACEI = angiotensin-converting-enzyme inhibitor, ARB = angiotensin II receptor blocker, ASA = American Society of Anesthesiologists' physical status score, BMI = body mass index, HDL = high-density lipoprotein, IQR = interquartile range, LDL = low-density lipoprotein, MAP = mean arterial blood pressure, SD = standard deviation.*

**Changes in the metabolites 24 hours postoperatively**

The AbsoluteIDQ® p180 Kit allows to identify 188 different metabolites: 21 amino acids, 21 biogenic amines, one carbohydrate molecule, 40 (acyl-) carnitines, 14 lysophosphatidylcolines, 76 phosphatidylcholines and 15 sphingomyelins. In this study, 103 of these metabolites and 20 metabolic ratios were included in the final analysis. All (acyl-) carnitines were excluded as they had been discussed in a separate paper by the same authors (Kasepalu et al., 2020b). Forty-five more metabolites were excluded, as >33% of their pre- or postoperative values were < LOD.

The baseline values of the analysed metabolites were similar in both groups (Table 12). There were no statistically significant differences between the groups in changes in the metabolites 24 h postoperatively (Table 12). In quantitative enrichment analysis, none of the metabolic pathways in SMPDB or KEGG databases were found to differ significantly in their response due to RIPC.

**Table 12.** List of the analysed metabolites and comparison of their baseline and postoperative values between the remote ischaemic preconditioning (RIPC) and sham groups in a randomised trial to assess the effects of a single episode of RIPC on metabolome in patients undergoing vascular surgery.

| *Metabolic group and metabolites* | *Baseline comparison* | *Change 24 h postoperatively* |
|---|---|---|
| **Amino acids (*n* = 19)**<br>Ala, Arg, Cit, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Orn, Phe, Pro, Ser, Thr, Trp, Tyr, Val | NS | NS |
| **Biogenic amines (*n* = 7)**<br>ADMA, Creatinine, Kynurenine, Serotonin, Spermine, Taurine, total DMA | NS | NS |
| **Glycerophospholipids (*n* = 62)** | NS | NS |
| **Sphingolipids (*n* = 14)**<br>SM(OH)C14:1, SM(OH)C16:1, SM(OH)C22:1, SM(OH)C22:2, SM(OH)C24:1, SMC16:0, SMC16:1, SMC18:0, SMC18:1, SMC20:2, SMC24:0, SMC24:1, SMC26:0, SMC26:1 | NS | NS |
| **Hexoses (*n* = 1)**<br>H1 | NS | NS |
| **Metabolic ratios (*n* = 20)**<br>(C2+C3)/C0, AAA, ADMA/Arg, BCAA, C2/C0, Cit/Arg, Cit/Orn, Essential AA, Fisher ratio, Glucogenic AA, Kynurenine/Trp, Nonessential AA, Orn/Arg, Putrescine/Orn, Serotonin/Trp, Total SM, Total SM-nonOH, Total SM-OH, Total SM-OH/Total SM-nonOH, Tyr/Phe | NS | NS |

*AA = Amino acids, AAA = Aminoadipic acid, ADMA = asymmetric dimethylarginine, Ala = Alanine, Arg = Arginine, BCAA = Branched chain amino acids, C0 = Carnitine, C2 = Acetylcarnitine, C3 = Propionylcarnitine, Cit = Citrulline, DMA = dimethylarginine, Gln = Glutamine, Glu = Glutamic acid, Gly = Glycine, H1 = hexose, His = Histidine, Ile = Isoleucine, Leu = Leucine, Lys = Lysine, Met = Methionine, nonOH = nonhydroxy, OH = hydroxy, Orn = Ornithine, Phe = Phenylalanine, Pro = Proline, Ser = Serine, SM = Sphingomyeline, SM(OH) = Hydroxysphingomyeline, Thr = Threonine, Trp = Tryptophan, Tyr = Tyrosine, Val = Valine.*

**Correlations of the metabolites with cardiac and kidney markers in the RIPC group**

In the RIPC group, there were no significant correlations between change in amino acid levels and change in heart injury markers. Regarding biogenic amines, change in total DMA level was positively correlated with shift in hs-TnT ($r$ = .496, $p$ < .001). Changes in different glycerophospholipids and sphingolipids did not correlate with changes in cardiac markers. Among the metabolic ratios, change in the kynurenine/tryptophan ratio showed positive linear correlation with change in hs-TnT ($r$ = .570, $p$ < .001) and change in NT-proBNP ($r$ = .552, $p$ < .001). These correlations were only seen in the RIPC group.

In the RIPC group, there were no significant correlations of change in amino acids, biogenic amines, glycerophospholipids, or sphingolipids with change in kidney injury markers. However, among the metabolic ratios, there was a significant positive linear correlation of change in the kynurenine/tryptophan ratio with cystatin C ($r$ = .534, $p$ < .001) and beta-2-microglobulin ($r$ = .504, $p$ < .001). These correlations were not observed in the sham group.

**Correlations of the metabolites with heart and kidney markers in the sham group**

In the sham group, no significant correlations were detected between alterations in amino acids and heart and kidney injury markers. Nor were there significant correlations between change in biogenic amines and change in hs-TnT or NT-proBNP. However, there was a significant positive linear correlation between change in total DMA and the change in creatinine ($r$ = .462, $p$ = .001) and beta-2-microglobulin ($r$ = .491, $p$ < .001).

There occurred no significant correlation of different glycerophospholipid and sphingolipid levels with concomitant changes in heart and kidney injury markers. Among the metabolic ratios, there was a significant positive linear correlation between change in the ADMA/Arg ratio and change in hs-TnT ($r$ = .527, $p$ < .001), between change in the Cit/Arg ratio and change in beta-2-microglobulin ($r$ = .500, $p$ < .001) and, between change in the putrescine/ornithine ratio and change in creatinine ($r$ = .615, $p$ < .001).

### 5.4.3. Summary of metabolomic findings across the studies (Papers III–IV)

Across two sham-controlled studies conducted in distinct clinical contexts, RIPC was not associated with widespread or sustained metabolic remodelling. In LEAD patients with IC undergoing daily RIPC for 28 days (Paper III), no statistically significant alterations in serum or skeletal muscle metabolites were observed. Specifically, no significant group × time interactions were detected for any metabolites in either serum or skeletal muscle (FDR > .05 for all). Furthermore, principal component analysis of both serum and muscle metabolite profiles showed no significant association between the first ten principal components and

treatment group (RIPC vs. sham), indicating comparable global metabolic profiles between groups.

In the setting of elective vascular surgery (Paper IV), a single episode of RIPC did not significantly alter serum metabolites 24 hours postoperatively. However, there was a positive correlation of change in the kynurenine/tryptophan ratio with change in hs-troponin T ($r = .570$, $p < .001$), NT-proBNP ($r = .552$, $p < .001$), cystatin C ($r = .534$, $p < .001$) and beta-2-microglobulin ($r = .504$, $p < .001$) only in the RIPC group. Prior analyses of the same patients had demonstrated reduced perioperative leakage of cardiac and renal injury biomarkers with RIPC, indicating that RIPC may have cardioprotective and nephroprotective effects in these settings (Kasepalu et al., 2020a; Kepler et al., 2020).

# 6. DISCUSSION

## 6.1. Summary of principal findings (Papers I–IV)

This PhD thesis evaluated the effects of repeated RIPC on functional capacity and a broad range of vascular and biochemical-metabolic biomarkers in LEAD patients with IC using a sham-controlled randomised design. The principal finding was that 28 consecutive days of daily RIPC did not result in clinically meaningful improvements in walking performance or QoL compared with a sham intervention (Paper I). The absence of significant changes in arterial stiffness, OS and inflammation biomarkers, as well as markers of cardiac and renal injury supported these neutral clinical findings (Paper II). Furthermore, targeted metabolomic analyses revealed no evidence of widespread metabolic remodelling in either serum or skeletal muscle following repeated RIPC (Paper III). Complementary metabolomic data from a vascular surgery setting demonstrated that a single episode of RIPC was similarly not associated with major global metabolic shifts (Paper IV).

## 6.2. Repeated RIPC does not improve walking distance or quality of life in IC (Paper I)

This randomised, sham-controlled, double blind trial demonstrated that home-based four week RIPC was safe, feasible and associated with high adherence. Despite excellent compliance, RIPC did not improve MWD, ICD and TRC compared with sham.

Few studies have assessed the effects of repeated RIPC on patients with LEAD. The first similar study was conducted by Ahmed et al. (2019), when patients with stable IC were randomly allocated to receive RIPC alone, SET alone, RIPC plus SET or to a control group, where the individuals received standard advice and risk factor modification over 28 days. The RIPC, SET and RIPC plus SET groups demonstrated statistically significant improvements in ICD and ABI within 30 days. However, there were no statistically significant changes in the results of the 6-minute walking test. Balin & Kivrak (2019) demonstrated that patients receiving repeated RIPC for 28 days showed an increase in MWD and ICD, as well as a decrease in TRC. However, the interpretation of these findings is complicated by methodological differences between the studies, including a lack of rigorous sham control and potentially compromised blinding, as in some studies, patients had to use a manual BP cuff to induce RIPC at home.

These early promising results were not replicated in the present study. The failure to show benefit of RIPC compared with sham intervention can be attributed to several factors. First, in the light of previous studies (Ahmed et al., 2019; Balin & Kivrak, 2019; Delagarde et al., 2015; Loo et al., 2022; Saes et al., 2013) and our own, the actual treatment effect of RIPC is probably smaller than that what was expected in the power calculation (20% improvement in MWD). Because the effect size was relatively small, the number of study participants may

have been insufficient to demonstrate a treatment effect. However, the median difference achieved between the groups in our current study may not be clinically relevant. Second, intra- and interpersonal variance in walking distances were underestimated. Exclusion of significant outliers could have reduced heterogeneity and large variance in the walking distances, but a potentially important group of patients would have been eliminated. All patients who completed the study were included in the analysis and there were no crossovers. Third, the lack of difference between the groups may be due to considerable improvement in the IC parameters in the sham group. The placebo effect can significantly affect non-pharmacological therapies and patient reported outcomes such as MWD and ICD. This signifies that blinding was achieved in this trial, which could also explain the excellent compliance in the sham group. It is plausible that expectations associated with participation in a novel intervention enabled some patients to tolerate pain better and increase activity. Furthermore, considering that both groups improved significantly over 28 days (significant time effect, $p = .001$ for MWD), this suggest that repeated treadmill testing itself may act as a form of exercise therapy. Since patients performed treadmill tests at baseline and follow-up, and the study did not control for changes in daily activity, the observed time effect could reflect an exercise/testing confound. Also, there was a trend for spontaneous improvement among patients with IC and, although they were considered stable on inclusion, this may not have been the case for all of them (Figure 2). However, this may not be as relevant in the context of this study since all patients had LEAD for a median of five years. Additionally, randomisation would most probably have equally divided spontaneous improvement between both groups.

Considering that the primary outcome of the trial was negative, and repeated measures ANOVA did not reveal a statistically significant group by time interaction effect, there is a high likelihood that repeated RIPC does not confer a clinically meaningful benefit in IC. Thus, as of now, there is no convincing evidence of the utility of RIPC to support its implementation into daily clinical practice. The lack of meaningful change in walking distances is supported by the lack of change in QoL, with an average change of 0 points in the VascuQoL-6 score in both groups.

## 6.3. Neutral vascular and biochemical biomarker responses suggest limited systemic conditioning in IC (Papers II, V)

To the best of our knowledge, this is the first randomised sham-controlled clinical trial that comprehensively assessed the effects of 28-day repeated RIPC on biomarkers of arterial stiffness, organ damage and OS in LEAD patients with IC. Findings from this trial suggest that repeated RIPC does not significantly influence these biomarkers in such patient cohort.

There are several possible reasons why RIPC did not yield measurable effects in this trial. First, the baseline levels of heart (hs-TnT, NT-proBNP), kidney (creatinine, urea, β2 microglobulin, NGAL, KIM-1), general inflammation (IL-6, IL-18) and arterial stiffness (cf-PWV) biomarkers were low in most patients; therefore, further reduction was unlikely to occur. At the same time, hs-CRP and MPO levels were elevated, indicating increased cardiovascular risk, but repeated RIPC had no effect on these markers. While RIPC is believed to mitigate a systemic inflammatory response and OS (Orlandi et al., 2021; Sun et al., 2023), this was not supported by our current findings. Minor changes, such as a slight reduction in diastolic BP and a modest increase in NGAL, were observed but did not achieve statistical significance after correcting for multiple comparisons (Tables 5 and 6). Taken together, these findings suggest that repeated RIPC does not induce meaningful systemic vascular conditioning or end-organ protection in LEAD patients with IC, at least within the timeframe and population studied.

## 6.4. RIPC is not associated with widespread metabolomic remodelling in IC (Papers III–IV)

Across two sham-controlled studies conducted in distinct clinical contexts, RIPC was not associated with widespread or sustained metabolic remodelling. In LEAD patients with IC undergoing repeated daily RIPC for 28 days (Paper III), no major metabolic adaptations were observed in either serum or skeletal muscle. PCA similarly revealed substantial overlap between the RIPC and sham groups, indicating broadly similar metabolomic profiles. These findings parallel the absence of functional improvement and neutral biomarker responses, indicating that repeated RIPC does not induce durable metabolic conditioning in IC.

Similarly, in the setting of elective major vascular surgery (Paper IV), targeted serum metabolomic profiling performed preoperatively and 24 hours postoperatively did not demonstrate global differences between the RIPC and sham groups. However, within the RIPC group, changes in the kynurenine-tryptophan ratio were significantly correlated with changes in cardiac and renal injury markers, including hs-TnT, NT-proBNP, cystatin C and β2-microglobulin. Prior analyses of the same patients had demonstrated reduced perioperative leakage of cardiac and renal injury biomarkers with RIPC, supporting that RIPC may play a role in cardioprotective and nephroprotective effects in these settings (Kasepalu et al., 2020a; Kepler et al., 2020). Consistent with this, the kynurenine pathway has been repeatedly implicated in RIPC-mediated protection, with experimental evidence suggesting that kynurenine exerts vasodilatory and cardioprotective effects (Bakhta et al., 2020; Chao de la Barca et al., 2016). While causality cannot be inferred, these findings suggest that the kynurenine pathway may play a role in RIPC-associated cardio- and nephroprotective effects initiated by a single RIPC episode prior to pronounced ischaemic stress, such as major vascular surgery.

Since its discovery by Murry et al. (1986), the concept of RIPC has been established in animal models, but its translation into clinical practice has been

challenging, with ongoing efforts to establish its role in different CVDs and patient populations. In the context of IC, RIPC has shown beneficial effects on skeletal muscle in animal models (Eberlin et al., 2008; Khalifa et al., 2023; Kocman et al., 2015; Mansour et al., 2012; Park et al., 2016), in smaller trials in healthy individuals (Barbosa et al., 2015; Niespodziński et al., 2021; Surkar et al., 2020), and in a few positive trials addressing IC (Ahmed et al., 2019; Balin & Kivrak, 2019; Loo et al., 2022). Based on our results, however, translating these protective effects to real-world patients appears challenging, as responses to RIPC may be attenuated by common comorbidities, medications and lifestyle factors (Bell et al., 2022). This has led to the concept of a "cardioprotective ceiling", whereby endogenous (spontaneous episodes of ischaemia and reperfusion in the affected leg) or treatment-induced protective pathways may already be activated, limiting the additional benefit achievable with further conditioning stimuli (Hummitzsch et al., 2021). Additionally, patients with IC may already be "hyperconditioned," a phenomenon proposed as a potential explanation for reduced responsiveness, possibly related to collagen injury and reduced skeletal muscle plasticity (Whittaker & Przyklenk, 2014).

The current metabolomic analyses had some limitations. First, the study was exploratory in nature, and the number of metabolites analysed was large relative to the sample size, particularly for skeletal muscle ($n$ = 16), limiting statistical power after multiple testing correction. Some statistically insignificant, but potentially biologically meaningful effects may have been missed. For example, 112 skeletal muscle metabolites, sums and ratios showed nominally significant ($p < 0.05$) changes in a consistent direction (Table 9), including increases in acylcarnitines, lipid species (phospholipids, ceramides, triglycerides) and metabolites related to energy and amino acid metabolism. This finding may suggest RIPC-induced metabolomic adaptation, which may be consistent with previous preclinical studies, in which, at the level of skeletal muscle, RIPC has been shown to improve mitochondrial function (Khalifa et al., 2023; Mansour et al., 2012), protect against ischaemia-reperfusion injury (Eberlin et al., 2008; Kocman et al., 2015), and upregulate genes involved in antioxidant defence and endoplasmic reticulum stress responses (Park et al., 2016). However, even if there was some metabolomic adaptation in skeletal muscle in response to RIPC, the effect was likely subtle and insufficient to translate into improvements in complex clinical outcomes, such as MWD, in IC. This consistent trend in muscle metabolites was unlikely to be solely due to technical factors. Muscle metabolites were normalised to the total protein content of each sample. Baseline and follow-up biopsies had comparable protein content, making it improbable to be a normalisation error. Additionally, all the samples were stored under the same conditions and analysed simultaneously at the end of the trial. Baseline and follow-up samples were run on the same 96-well plate, aiming to avoid batch- or run-order artefacts. Consequently, this trend seen in muscle metabolites warrants validation in adequately powered studies. Second, we used the MxP® Quant 500 kit, which provides broad coverage of targeted metabolites and is well-suited to a hypothesis-free approach when the aim is to identify potential biomarkers. At the same time,

using this metabolomics kit poses certain risks, including incomplete coverage of potentially relevant pathways, such as energy metabolism, potentially leaving some pathways on which RIPC could act uncovered. Despite this, the choice of this kit was justified because very little is known about the potential effects of RIPC on the metabolome in patients with IC, and targeting a broader profile would help identify novel directions to explore. Additionally, using this well-known kit makes different studies comparable and helps discover common patterns. Third, the median time from end of treadmill to muscle biopsy was around 10–15 minutes (Table 7), although all efforts were made to obtain the specimen as soon as possible after the treadmill test, the preparation for the procedure took time, and short-lived ischaemia-induced metabolite shifts would have had time to decay, potentially reducing the chance to detect the effect of RIPC. Fourth, serum and muscle metabolites were measured at different time points: serum was collected at rest, while muscle samples were obtained immediately after the treadmill test. As a result, reliable correlations between serum and muscle metabolite levels could not be assessed.

Taken together, the metabolomic findings indicate that repeated RIPC does not induce substantial changes in systemic or local metabolome in patients with IC. There may be some subtle adaptations locally at the level of skeletal muscle. Given the RIPC's neutral effect on MWD and minimal changes on metabolome, metabolic remodelling is unlikely to represent a significant mechanism of benefit of repeated RIPC in IC.

## 6.5. Biological limitations of repeated RIPC in IC (Papers I–V)

The effects of RIPC appear to be highly dependent on the ischaemic context in which it is applied. RIPC was originally discovered to attenuate acute ischaemia-reperfusion injury, a setting characterised by abrupt, profound ischaemia followed by reperfusion and predictable biomarker release (Heusch, 2015; Heusch et al., 2015). In such circumstances, like in acute myocardial infarction or cardiac or vascular surgery involving arterial clamping, the timing of RIPC relative to the ischaemic insult can be optimised, and the protective effects may thus be more readily detectable (Kasepalu et al., 2020a; Kepler et al., 2019). In contrast, IC represents a fundamentally different pathophysiological state, marked by its chronic status with repetitive episodes of ischaemia-reperfusion during daily ambulation. In this setting, ischaemic stimuli are typically submaximal and temporally unpredictable, and the timing of RIPC relative to ischaemic insult may not have been optimal to achieve maximal benefit. The temporal delay and unpredictable nature of spontaneously occurring ischaemia-reperfusion episodes in IC may have contributed to the neutral results of this study. There was no specific sustained ischaemic event on which RIPC could act. On the other hand, the biphasic nature of RIPC-induced protection comprising an early phase lasting

several hours and a delayed phase emerging after 24–48 hours provides a theoretical rationale for repeated daily application (Heusch, 2015; Heusch et al., 2015; Loukogeorgakis et al., 2005). The intention of repeated RIPC was to maintain a persistent state of protection over time.

Another proposed explanation for the lack of efficacy of repeated RIPC in IC is that chronically ischaemic skeletal muscle may already be in a state of background or endogenous preconditioning. Recurrent exercise-induced ischaemia can activate protective pathways similar to those triggered by RIPC, thereby diminishing the incremental benefit of an externally applied conditioning stimulus. In this context, RIPC applied to a relatively small muscle mass in the upper limb may be insufficient to further modulate the biology of large, chronically affected lower-limb muscle groups.

The dose, delivery and biological threshold effects further complicate the interpretation of repeated RIPC. The optimal intensity, frequency and duration of repeated RIPC remain unknown, and no study has compared different protocols. Evidence from large neurological trials indicates that even prolonged daily conditioning may yield neutral overall results, with only modest signals of benefit in highly adherent subgroups (Blauenfeldt et al., 2022; Blauenfeldt et al., 2023; Blauenfeldt et al., 2024; Chen et al., 2022; Hou et al., 2022). These findings underscore the difficulty of maintaining an effective RIPC stimulus over time, as evidenced by poor adherence to repeated RIPC protocols lasting up to one year (Hou et al., 2022), and suggest that bilateral, as opposed to unilateral, upper-limb RIPC may generate detectable benefit of RIPC in chronic settings (Chen et al., 2022).

Additionally, LEAD patients with IC often receive comprehensive guideline-directed medical therapy, including statins, antiplatelet agents, renin-angiotensin system inhibitors, insulin or metformin and other glucose-lowering therapies. Many of these treatments activate cytoprotective pathways overlapping with those of RIPC and may independently reduce arterial stiffness and cardiovascular risk, thereby raising the threshold required for additional benefit from RIPC (Bell et al., 2022; Heusch et al., 2013; Heusch et al., 2015; Laurent et al., 2014; Tropeano et al., 2006). Advanced age and common comorbidities, such as hypertension, dyslipidaemia, diabetes and chronic kidney disease, are also known to attenuate both neural and humoral components of RIPC signalling (Ferdinandy et al., 2014; Kleinbongard et al., 2020). In particular, diabetes mellitus has been shown to impair the efficacy of RIPC by disrupting signal transmission pathways (Hansen et al., 2019; Torregroza et al., 2021). Indeed, approximately 30% of patients in the RIPC group in the present trial had diabetes. Collectively, this raises the question of "cardioprotective ceiling": when endogenous or treatment-induced protective pathways are already activated, additional conditioning stimuli may fail to elicit further benefit, which in turn results in an apparent ceiling effect (Hummitzsch et al., 2021). Additionally, LEAD patients with IC may already be "hyperconditioned", a phenomenon which may lead to collagen injury (primary fibre breakage) that limits skeletal muscle plasticity and contributes to the "ceiling" effect (Whittaker & Przyklenk, 2014).

Taken together, these considerations suggest that repeated RIPC may be biologically constrained in IC by background preconditioning, limited skeletal muscle plasticity, pharmacological ceiling effects and attenuated signalling capacity in older, comorbid patients.

## 6.6. Clinical implications: should RIPC be pursued in IC?

The findings of this thesis have important implications for the clinical applicability of RIPC in LEAD patients with IC. Despite strong biological plausibility and encouraging results from preclinical studies and early pilot trials (Ahmed et al., 2019; Balin & Kivrak, 2019), repeated daily RIPC did not improve walking performance, QoL or measurable vascular, biochemical, or metabolic outcomes in this trial. These results do not support the routine use of repeated RIPC as a therapeutic intervention for IC.

From a clinical perspective, effective treatments for IC must deliver clear and meaningful functional benefits, as symptom relief and improvement in walking performance are among the key treatment objectives. Given the elevated cardiovascular risk associated with LEAD (Caro et al., 2005; Deihm et al., 2009), an ideal intervention would also favourably influence systemic vascular health and long-term prognosis. In this regard, SET combined with guideline-directed medical therapy remains the most effective non-invasive treatment, with consistent evidence demonstrating clinically relevant improvements in walking performance and QoL (Mazzolai et al., 2024; Nordanstig et al., 2024). The absence of comparable benefits with repeated RIPC indicates that this method should not be considered an alternative or substitute for established therapies.

The lack of detectable effects on arterial stiffness, OS, end-organ injury and skeletal muscle metabolism further limits the rationale for adopting repeated RIPC in routine clinical practice. Notably, the consistency of neutral findings across multiple outcome domains suggests that the observed lack of efficacy is unlikely to be explained solely by the insensitivity of the chosen outcome measures. Instead, the data indicate that repeated RIPC does not induce sufficient biological adaptation to translate into a clinically meaningful benefit in patients with IC. Clinical management in IC should continue to prioritise interventions with proven efficacy. At the same time, future research on RIPC should focus on clearly defined contexts in which biological plausibility and clinical relevance are strongest**.**

## 6.7. Strengths and limitations

**Strengths**

A major strength of this trial was the prioritisation of patient-centred clinical outcomes, namely walking performance and QoL. These outcomes directly reflect the symptomatic burden of IC and are central to clinical decision-making. While biomarker outcomes can provide valuable mechanistic insight, they often have

limited direct translatability to patient benefit. For example, modest reductions in markers of biochemical injury do not necessarily translate into reductions in clinical events or possibly also mortality. Positioning biomarkers as secondary outcomes, therefore, strengthens the study's clinical relevance.

Another key strength was the use of a standardised, automated RIPC device, which ensured consistent delivery of the intervention and minimised operator variability. Treatment adherence was objectively monitored via the device application and was high, with only a few missed sessions. This high level of compliance strengthens confidence that the neutral findings were not attributable to inadequate exposure to the intervention.

Finally, the randomised, sham-controlled, double-blind design represents a major methodological strength in a field where placebo effects and expectation bias are particularly relevant, especially for symptom-based outcomes such as MWD.

**Limitations**

Several limitations should be acknowledged. First, the sample size calculation was based on an expected 20% improvement in MWD in the RIPC group vs. 2% in the sham group. However, the actual treatment effect of RIPC is probably smaller than that was expected in the power calculation, therefore the number of study participants may have been insufficient to demonstrate treatment effect. Still, a larger study may have demonstrated a small difference between the groups; however, this difference still may not be clinically relevant. In this study, repeated RIPC improved MWD by 24 m, whereas SET has been shown to improve MWD by approximately 180 m in a large meta-analysis (Fakhry et al., 2012). Regarding biomarker and metabolomic outcomes, some subtler, but potentially biologically relevant effects, may have been dismissed due to a lack of power (Sections 6.2, 6.3 and Paper V). The Bonferroni correction was applied in Paper II as a conservative approach given the clinical nature of the biomarker outcomes, while the Benjamini-Hochberg procedure was used in Paper III given the exploratory nature of the metabolomic analysis. Still, the absence of consistent signals across multiple outcome domains (clinical, vascular and biochemical-metabolic) argues against a clinically meaningful benefit.

Second, although the sham device was visually identical to the autoRIC device and had the same sound and vibration effects, the cuff was not inflated during the sham protocol, potentially allowing some participants to deduce their group allocation. However, since adherence to the treatment was excellent in both groups, this could not have been a substantial number of patients.

Third, the results cannot be generalised to all patients with IC, as the study included only male participants aged 18–80 years. Women were excluded to maintain a homogeneous cohort in a relatively small study. Although LEAD is equally prevalent in women and men (Nordanstig et al., 2024), men are substantially overrepresented in the clinical practice in our country. Furthermore, some early studies suggested potential sex differences in RIPC responsiveness,

with weaker effects reported in women (Heinen et al., 2018); however, in larger clinical trials, no significant sex differences have been reported.

Fourth, approximately 30% of patients in the RIPC group had diabetes mellitus, which may have contributed to the lack of observed benefit, as diabetes is known to attenuate conditioning responses (Hansen et al., 2019; Torregroza et al., 2021). However, diabetic participants in this trial had well-controlled disease, and patients with diabetic polyneuropathy were excluded, given evidence that neuropathy disrupts neural signal transmission essential for the efficacy of RIPC (Heusch et al., 2015). In addition, in large clinical trials with prespecified subgroup analyses, the presence of diabetes has not affected RIPC (Hausenloy et al., 2015; Hausenloy et al., 2019). While diabetes and other comorbidities may raise the biological threshold for conditioning, excluding such patients entirely would reduce generalisability and fail to reflect real-world clinical populations.

Finally, the study population may also reflect some selection bias, as the participants were likely more motivated and less frail than the broader IC population. The very high adherence observed may therefore not be fully representative of routine clinical settings.

# 7. CONCLUSIONS

1. Twenty-eight consecutive days of RIPC did not improve symptoms of IC, assessed by the change in MWD, ICD, TRC and QoL. Daily RIPC was safe and showed high adherence to treatment by the patients.
2. Twenty-eight consecutive days of RIPC had no significant effect on arterial stiffness, and biomarkers of inflammation, OS and end-organ damage in patients with IC. Repeated RIPC in patients with LEAD and stable IC did not provide organ protection.
3. Repeated RIPC did not result in significant changes in systemic or local metabolic profiles in patients with IC. Additionally, a single episode of preoperative RIPC did not significantly affect metabolome 24 hours after vascular surgery. The kynurenine pathway may play a role in RIPC-associated cardio- and nephroprotective effects in patients undergoing elective vascular surgery.

# 8. IMPLICATIONS FOR FUTURE RESEARCH

The key aspects that should be evaluated in future research include:

**1. Optimal duration, dosing and adherence of repeated RIPC**. The chronic temporal characteristics of RIPC-induced protection remain poorly defined. No study has systematically established the optimal duration, frequency, or cumulative "dose" of repeated RIPC. Treatment adherence and dosing are closely interrelated, with longer protocols associated with poorer adherence. Therefore, future clinical studies should clarify how protocol intensity and duration influence both biological effects and long-term adherence.

**2. Biomarkers of RIPC responsiveness**. Identification of reliable biomarkers of RIPC response would substantially advance the field. Such biomarkers could improve mechanistic understanding of RIPC effects, help identify individuals most likely to benefit ("responders"), and support personalised optimisation of RIPC dose and duration.

**3. Patient selection and disease stage**. Further research is needed to determine whether specific patient subgroups within LEAD are more responsive to RIPC. Patients with less advanced IC (Fontaine IIa) may retain greater skeletal muscle plasticity and functional reserve than those with Fontaine IIb disease, in whom established myopathy may limit responsiveness.

**4. Combining RIPC with SET**

Combining RIPC with SET or home-based walking exercise by applying RIPC immediately prior to exercise-induced ischaemia may enhance its therapeutic potential. This strategy is conceptually attractive because SET remains the most effective non-invasive treatment for IC; however, access to SET is limited in many countries. Integrating RIPC with home-based walking programmes may augment exercise-induced adaptations, allow shorter or more flexible protocols, and improve adherence and real-world applicability.

# 9. SUMMARY IN ESTONIAN

## Kaugisheemilise eelkohastamise mõju funktsionaalsele võimekusele, vaskulaarsetele ja biokeemilis-metaboolsetele biomarkeritele alajäseme arterite haigusega patsientidel

Alajäseme arterite haigus (AAH) on ateroskleroosi üks pahaloomulisemaid kliinilisi väljendusvorme, mille korral alajäsemete arterite ahenemine või sulgus põhjustab jalgade verevarustuse häireid. Haigus võib kulgeda asümptomaatiliselt, kuid sageli avaldub vahelduva lonkamisena, rahuolekuvalude, haavandite või gangreenina. Maailmas põeb AAH ligikaudu 230 miljonit inimest ning haiguse levimus on viimastel aastakümnetel kasvanud nii arenenud riikides kui ka arengumaades.

AAH patsientidel esineb sageli polüvaskulaarne ateroskleroos, mistõttu on neil oluliselt suurenenud südameinfarkti, insuldi ja kardiovaskulaarse suremuse risk. Seetõttu on haiguse ravi aluseks agressiivne riskifaktorite kontroll, sealhulgas suitsetamisest loobumine, vererõhu ja lipiidide taseme korrigeerimine, trombotsüütide agregatsiooni pärssiv ravi ning elustiilimuutused. Revaskulariseerimist rakendatakse juhul, kui sümptomid oluliselt piiravad elukvaliteeti või ohustavad jäseme eluvõimelisust, kuid tegemist on invasiivse meetodiga, millega võivad kaasneda vajadus kordusprotseduuri järgi või tüsistuste risk.

Käesolev doktoritöö keskendub AAH ühele sagedasemale kliinilisele vormile – vahelduvale lonkamisele. Vahelduv lonkamine on tingitud alajäseme verevarustushäirest koormusel, põhjustades käimisel alajäsemevalu, mis sunnib patsienti liikumist katkestama ning viib sageli füüsilise aktiivsuse vähenemise, autonoomia kaotuse ja elukvaliteedi languseni. Ravivõimalused on piiratud: kuigi juhendatud kõnniteraapia on efektiivne, ei ole see paljudes riikides, sealhulgas Eestis, laialdaselt kättesaadav. Seetõttu on vajalik uute, ohutute ja laialdaselt rakendatavate ravistrateegiate uurimine.

Vahelduva lonkamise üheks potentsiaalseks uudseks ravialaseks lähenemiseks on kaugisheemiline eelkohastamine (KIE), mille käigus tekitatakse lühiajalised isheemia-reperfusiooni episoodid eemal asuvas koes (tavaliselt ülajäsemes), et aktiveerida organismi endogeensed kaitsemehhanismid. KIE on näidanud kaitsvat toimet südame ja neerude suhtes ägedates isheemia-reperfusiooni olukordades, kuid selle roll kroonilistes seisundites, sealhulgas vahelduvas lonkamises, on ebaselge.

Senised KIE uuringud vahelduva lonkamisega patsientidel on olnud väikesemahulised ning metoodiliselt heterogeensed. Sageli on puudunud adekvaatne pimendamine ning KIE on teostatud manuaalse vererõhumansetiga, mistõttu ei ole olnud võimalik objektiivselt hinnata sekkumise korrektsust ja ravisoostumust. Uuringute tulemused on olnud vastukäivad, mistõttu on vajalikud täiendavad, näivsekkumisega (ingl. *sham*)-kontrollitud kliinilised uuringud.

Käesoleva doktoritöö eesmärk oli hinnata 28 päeva kestva igapäevase KIE mõju vahelduva lonkamisega patsientidele, kasutades automatiseeritud KIE aparaati ja

topeltpimendatud randomiseeritud kontrollitud uuringudisaini. Hinnati KIE mõju kliinilistele tulemusnäitajatele (maksimaalne ja valuvaba käimisvahemaa, sümptomite taandumise aeg, elukvaliteet, meetodi turvalisus, ravisoostumus), veresoonte jäikusele, organkahjustuse, põletiku ja oksüdatiivse stressi biomarkeritele ning seerumi ja skeletilihase metaboloomile.

Uuringusse kaasati 42 meespatsienti vanuses 18–80 aastat, kellel oli stabiilne AAH ja vahelduv lonkamine. Patsiendid randomiseeriti KIE- või näivsekkumise rühma ning protseduurid viidi läbi ambulatoorsetes tingimustes 28 päeva jooksul. Ravisoostumus oli väga hea ning sekkumine osutus ohutuks ja hästi talutavaks.

Uuringu tulemused näitasid, et 28-päevane KIE ei parandanud maksimaalset ega valuvaba käimisvahemaad, ei lühendanud sümptomite taandumise aega ega parandanud elukvaliteeti võrreldes näivsekkumisega. Samuti ei täheldatud KIE mõju arterite jäikusele, südame- ja neerukahjustuse biomarkeritele ega põletiku ja oksüdatiivse stressi näitajatele. Metaboloomika analüüsides ei ilmnenud olulisi süsteemseid ega lokaalseid metaboolseid muutusi.

Praegused tulemused viitavad sellele, et igapäevane KIE ei too vahelduva lonkamisega AAH patsientidel kaasa olulist kliiniliselt kasu ega mõjuta arterite funktsiooni, biokeemilisi ja ainevahetuse biomarkereid. Üheks võimalikuks selgituseks on asjaolu, et need patsiendid võivad olla juba krooniliselt „eelkohastunud“ korduvate alajäseme isheemia-reperfusiooni episoodide tõttu, mistõttu täiendav KIE stiimul ei anna lisandväärtust. Samuti võivad KIE efekti piirata kõrge vanus, kaasuvad haigused ja kaasaegne medikamentoosne ravi, mis aktiveerib sarnaseid tsütoprotektiivseid radasid.

Kokkuvõttes näitab käesolev doktoritöö, et KIE-l ei ole olulist mõju vahelduva lonkamise sümptomitele, arterite jäikusele ega biokeemilistele ja ainevahetusega seotud biomarkeritele. Nende tulemuste põhjal ei saa KIE rutiinset kasutamist vahelduva lonkamise ravis soovitada.

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# PUBLICATIONS

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Eerik K, Kasepalu T, Post H, Eha J, Kals M, Björck M, et al. Effects of repeated remote ischaemic preconditioning on arterial stiffness, organ damage, and oxidative stress in patients with intermittent claudication: a sham controlled randomised trial. Eur J Vasc Endovasc Surg. 2026;71:117–123.

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Eerik K, Kasepalu T, Eha J, Kilk K, Kals J. Remote ischemic preconditioning does not provoke metabolomic alterations in patients undergoing major vascular surgery: a randomized controlled trial. Atherosclerosis; 2023;379: S180–181.

Eerik K, Kasepalu T, Eha J, Ottas A, Kals J. Kaugisheemilise eelkohastumise mõju metaboloomikale ning neeru- ja südamekahjustuse markeritele veresoontekirurgias. 2022. Eesti Arst, 101: 21−21.

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2024 Scientific Award of Estonian Society of Cardiology
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| 2023 | „Research Methods for Clinical Trials – A Short Course in Design and Management of Clincial Trials“, 14–19 May 2023, Estonia (lectors from University College London) |
| 2022 | Salzburg Weill Cornell Seminar in Cardiology, 3–9 July 2022, Austria |
| 2019 | University of Helsinki, Helsinki Summer School (HSS), „Cognition, Communication and the Brain“ (6 ECTS), Finland |
| 2016–2018 | Lecturer of the project „Vaccination – a Healthier Future for Our Children“ |

# ELULOOKIRJELDUS


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**Hariduskäik:**

| | |
|---|---|
| 2021–... | Tartu Ülikool, Arstiteaduskond, Kirurgiakliinik, doktorantuur |
| 2019–... | Tartu Ülikool, Arstiteaduskond, Kardioloogia kliinik, residentuur |
| 10/2024–03/2025 | Cambridge'i Ülikool, külalisdoktorant |
| 2013–2019 | Tartu Ülikool, Arstiteaduskond, integreeritud bakalaureuse- ja magistriõpe, *cum laude* |
| 2011–2019 | Nõo Reaalgümnaasium |

**Teenistuskäik:**

| | |
|---|---|
| 04/2020–... | Tartu Ülikooli Kliinikum, Põhja-Eesti Regionaalhaigla ja Pärnu Haigla – arst-resident (kardioloogia) |
| 02/2023–09/2025 | Tartu Ülikool, kliinilise meditsiini instituut, kirurgiakliinik – vasoloogia nooremteadur |
| 04/2020–08/2023 | Tartu Kiirabi – üldarst |
| 12/2020–01/2022 | Tartu Ülikooli Kliinikum, pulmonoloogia Covid-19 osakond – üldarst |
| 08/2018–04/2019 | Mikkeli Keskhaigla – abiarst |
| 06/2017–07/2018 | Ida-Tallinna Keskhaigla, erakorralise meditsiini osakond – abiarst |
| 07/2016–06/2017 | Tartu Ülikooli Kliinikum, 1. intensiivraviosakond – intensiivravi abiõde |
| 01/2014–06/2016 | Tartu Ülikooli Kliinikum, erakorralise kardioloogia osakond – intensiivravi hooldaja |

**TEADUSTÖÖ**

**Publikatsioonid:**


Eerik K, Kasepalu T, Post H, Eha J, Kals M, Björck M, et al. Effects of repeated remote ischaemic preconditioning on arterial stiffness, organ damage, and oxidative stress in patients with intermittent claudication: a sham controlled randomised trial. Eur J Vasc Endovasc Surg. 2026;71:117–123.

Eerik K, Kals M, Kals J. Possible effects of remote ischaemic pre-conditioning in patients with intermittent claudication. Eur J Vasc Endovasc Surg. 2025 [Articles in Press].

Eerik K, Kasepalu T, Post H, Eha J, Kals M, Kals J. Daily remote ischaemic preconditioning for intermittent claudication: a sham controlled randomised trial. Eur J Vasc Endovasc Surg. 2025;69:295–302.

Eerik K, Kasepalu T, Kuusik K, Eha J, Vähi M, Kilk K, et al. Effects of RIPC on the metabolome in patients undergoing vascular surgery: a randomized controlled trial. Biomolecules. 2022;12:1312.

Eerik K, Vibo R, Kreis A, Kõrv J. Insuldi revaskulariseeriva ravi hetkeseis Eestis. Eesti Arst 2018;97:240–246.

**Publitseeritud konverentside teesid:**

Post H, Paapstel K, Kilk K, Ööbik T, Eerik K, Kals J.Acute local metabolomic alterations in blood in intermittent claudication. Atheroclerosis. 2025:407: S119864.

Eerik K, Kasepalu T, Eha J, Kilk K, Kals J. Remote ischemic preconditioning does not provoke metabolomic alterations in patients undergoing major vascular surgery: a randomized controlled trial. Atherosclerosis; 2023;379: S180–181.

Eerik K, Kasepalu T, Eha J, Ottas A, Kals J. Kaugisheemilise eelkohastumise mõju metaboloomikale ning neeru- ja südamekahjustuse markeritele veresoontekirurgias. 2022. Eesti Arst, 101: 21−21.

**Kuulumine ühendusteesse:**

Eesti Kardioloogide Selts, Euroopa Kardioloogide Selts, Eesti Hüpertensiooni Ühing, Eesti Arstide Liit

**Teaduspreemiad ja tunnustused**

2024 Scientific Award of the Estonian Society of Cardiology
2022 Scientific Award of the Estonian Society of Cardiology
2022 The best e-poster oral presentation in the scientific conference of the Anniversary of the Faculty of Medicine
2019 North Estonia Medical Centre Award for the best scientific article published in the journal Eesti Arst in 2018
2018 Neuroconference Tartu 2018, 2nd place

**Täiendkoolitus**

2026 European Association of Cardiovascular Imaging (EACVI) sertifikatsioon täiskasvanute transtorakaalse ehhokardiograafia alal
2024 Euroopa Hüpertensiooni Ühingu suvekool, 15.–20. september 2024, Prantsusmaa
2025 Põhja-Balti doktorantide kohtumine (Soome Hüpertensiooni Ühingu koolitus), 7.–9. oktoober, Soome
2023 „Research Methods for Clinical Trials – A Short Course in Design and Management of Clincial Trials“, 14.–19. mai 2023, Eesti (lektorid University College Londonist)

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| 2022 | Salzburgi täiendusseminar arstidele (kardioloogia), 3.–9. juuli 2022, Austria |
| 2019 | Helsinki Ülikooli suvekool „Cognition, Communication and the Brain“ (6 EAP), Soome |
| 2016–2018 | Projekti „Vaktsineerimine – meie laste tervem tulevik“ lektor |

# DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

1. **Heidi-Ingrid Maaroos**. The natural course of gastric ulcer in connection with chronic gastritis and *Helicobacter pylori*. Tartu, 1991.
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