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A Randomized Control Trial Investigating the Effect of Different Treatment Strategies on Mitochondrial Function in Peripheral Arterial Disease: A Study Protocol

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ABSTRACT

Peripheral arterial disease encompasses different clinical symptoms, depending on the severity of the disease. In early stages, a walking-induced pain, known as intermittent claudication, is the leading clinical symptom. Repeating cycles of ischemia and reperfusion induce a typical myopathy, with mitochondria playing the key role within this pathophysiological condition. The aim of this study is to further evaluate the effects of different treatment strategies on mitochondrial function and overall cardiovascular outcomes within a randomized controlled trial. After inclusion, patients will be randomized into different study groups. Study group 1 will receive conservative treatment, while study group 2 will receive revascularization of underlying atherosclerotic lesions. Additionally, a healthy control group will be included. Muscle biopsies will be obtained from ischemic and nonischemic muscle regions, being defined by the anatomic localization of the atherosclerotic lesion, before initiation of treatment as well as after a time interval of 12 wk. Mitochondrial function and content will be evaluated using high-resolution respirometry and citrate synthase activity measurements. Cardiovascular outcomes will be determined by established protocols. This study is registered on ClinicalTrials.gov –NCT05644158. This study aims to gain further insights into the exact pathophysiological mechanism underlying mitochondrial dysfunction in peripheral arterial disease. The potential effects of mitochondrial regeneration within ischemic muscle regions following a conservative treatment approach will be compared to those reported after revascularization procedures. Additionally, correlation with cardiovascular outcome parameters and *in vivo* methods will provide a comprehensive approach to this research question.

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Introduction

Peripheral arterial disease (PAD), as a result of a chronic stenosis or occlusion of lower extremity arteries, results in malperfusion of affected extremities. Being one of several clinical manifestations of atherosclerosis, the burden of the disease is increasing with the rise of the elderly, affecting more than 200 million people worldwide.[1,2] Depending on clinical disease severity, PAD is categorized according to the Rutherford or the Fontaine classification systems.[3,4] PAD is often asymptomatic despite the loss of peripheral pulses; however, symptomatic patients describe a walking-induced pain as the leading symptom – known as intermittent claudication – in malperfused muscle regions. More severe stages of PAD are associated with ischemic rest pain and trophic lesions, defined as chronic limb-threatening ischemia and the potential need for amputation of the affected limb within the course of disease.[5] With a relatively low rate of major amputation in claudicants ranging from 1 to 3.3% reported by the Inter-Society Consensus for the Management of Peripheral Arterial Disease II consortium,[6] a meta-analysis published in 2016 described a rather more aggressive clinical course in a group of patients with initial mild forms of PAD (asymptomatic PAD and claudicants), leading to amputation rates of 4-27%.[7] Amputation remains the main fear of patients being affected by PAD. Therefore, the exact understanding of the pathophysiology of the diseases is mandatory in order to prevent such drastic measures. In claudicants, repeated cycles of ischemia and reperfusion cause a typical myopathy in affected muscle regions with changes at the cellular and subcellular level.[8,9] Using light and electron microscopy, these changes have been visualized and characterized by several authors.[10,11] Mitochondria are known to play a crucial role within this pathophysiological process.[12] In a recent review reporting details of mitochondrial alterations in PAD, mitochondriopathy was defined by three main mechanisms as follows: alterations within the mitochondrial respiratory chain itself, elevated release of reactive oxygen species and an increase of the mitochondrial calcium retention capacity, which is associated with enhanced apoptosis.[13] In previous studies, we demonstrated the ability of mitochondria to regenerate in terms of content as well as respiratory rates in symptomatic PAD patients after revascularization procedures.[14,15] For patients suffering from intermittent claudication, different treatment strategies are recommended in clinical guidelines ranging from conservative management with best medical treatment and exercise training for revascularization of the underlying atherosclerotic lesion.[5] The goal of this randomized controlled study is to evaluate the effect of conservative treatment (home-based monitored exercise training) in comparison to revascularization (endovascular or open surgery) on mitochondrial function in symptomatic PAD. With correlation of *in vivo* to *in vitro* methods for the evaluation of mitochondrial function and content, as well as parameters of cardiovascular risk, additional mechanistic information on ongoing pathophysiological mechanisms within affected muscle regions can be obtained.

Material and Methods

This is the study protocol of a randomized controlled trial, investigating the effect of different treatment strategies (conservative treatment versus revascularization) on mitochondrial function in patients with symptomatic PAD. The study was registered on ClinicalTrials.gov (NCT05644158), and ethical approval has been obtained by the ethics committee of the Medical University of Innsbruck, Austria (EK Nr. 1194/2019). All patients who will be included in the study will provide written consent, and withdrawal from the study will not affect any treatment decisions.

The description of the study protocol adheres to the Standard Protocol Items: Recommendations for Interventional Trials guidelines for reporting study protocols.[16]

Eligibility, inclusion, and exclusion criteria

Patients will be consecutively recruited at the outpatient clinic of the Department of Vascular Surgery, Medical University of Innsbruck. The inclusion criteria are unilateral symptomatic lower limb PAD (fontaine stage IIB; Rutherford stage 2 or 3) due to an isolated, flow-limiting atherosclerotic lesion of the superficial femoral artery (SFA). The exclusion criteria are contralateral symptomatic PAD (fontaine stage IIB – IV; Rutherford stage 3 -6), flow-limiting atherosclerotic lesions of the infrarenal aorta, iliac arteries, common femoral artery or deep femoral artery, a history of any prior revascularization procedure or related to PAD of the affected lower limb, any contraindication to exercise training, and allergy to local anesthesia. As the underlying isolated atherosclerotic lesion is located at the SFA, the gastrocnemius muscle is defined as the ischemic muscle region, whereas the vastus lateralis muscle is defined as the nonischemic muscle region. All patients will receive best medical treatment recommended for symptomatic PAD according to clinical guidelines, including medical treatment of modifiable cardiovascular risk factors as well as advice for lifestyle modification (e.g., smoking cessation).[5]

Randomization to different study groups

After screening for eligibility and obtaining written consent, eligible patients will be randomized into different study groups using a computer-based nonstratified 1:2 randomization process. Study group 1 will undergo conservative treatment with monitored home-based exercise training for a total of 12 wk (see Supplement 1 for details). Study group 2 will undergo interventional revascularization of the underlying flow-limiting atherosclerotic lesions of the SFA. Following clinical guidelines, lesions with a total length of less than 25 cm and a patent origin of the SFA at the femoral bifurcation will be treated by endovascular techniques (percutaneous transluminal angioplasty with or without stenting) (subgroup 2A). Lesions with a total length exceeding 25 cm with occlusion of the origin of the SFA at the femoral bifurcation will be treated by femoropopliteal bypass surgery (subgroup 2B). Additionally, a cohort of nonischemic control patients, undergoing planned

varicose vein surgery, will be included as a control group within this study. The study design is illustrated in Figure 1.

Primary and secondary outcome

The primary endpoint of the study is the change in mitochondrial function, assessed by *in vitro* high-resolution respirometry (HRR) and enzyme measurements as well as *in vivo* near-infrared retracted spectroscopy (NIRS) between baseline and the final measurement taken 12 wk after the allocated treatment modality. The secondary endpoint is the effect of different treatment modalities on systemic outcome measures for cardiovascular risk assessment at baseline and 12 wk after the allocated treatment modality. This will be evaluated by measuring flow-mediated dilatation (FMD) of the brachial artery, a standardized six-min walking test and the ankle-brachial index (ABI).

General cardiovascular assessment

A complete medical history, including demographic parameters (age, sex, and body mass index), cardiovascular risk

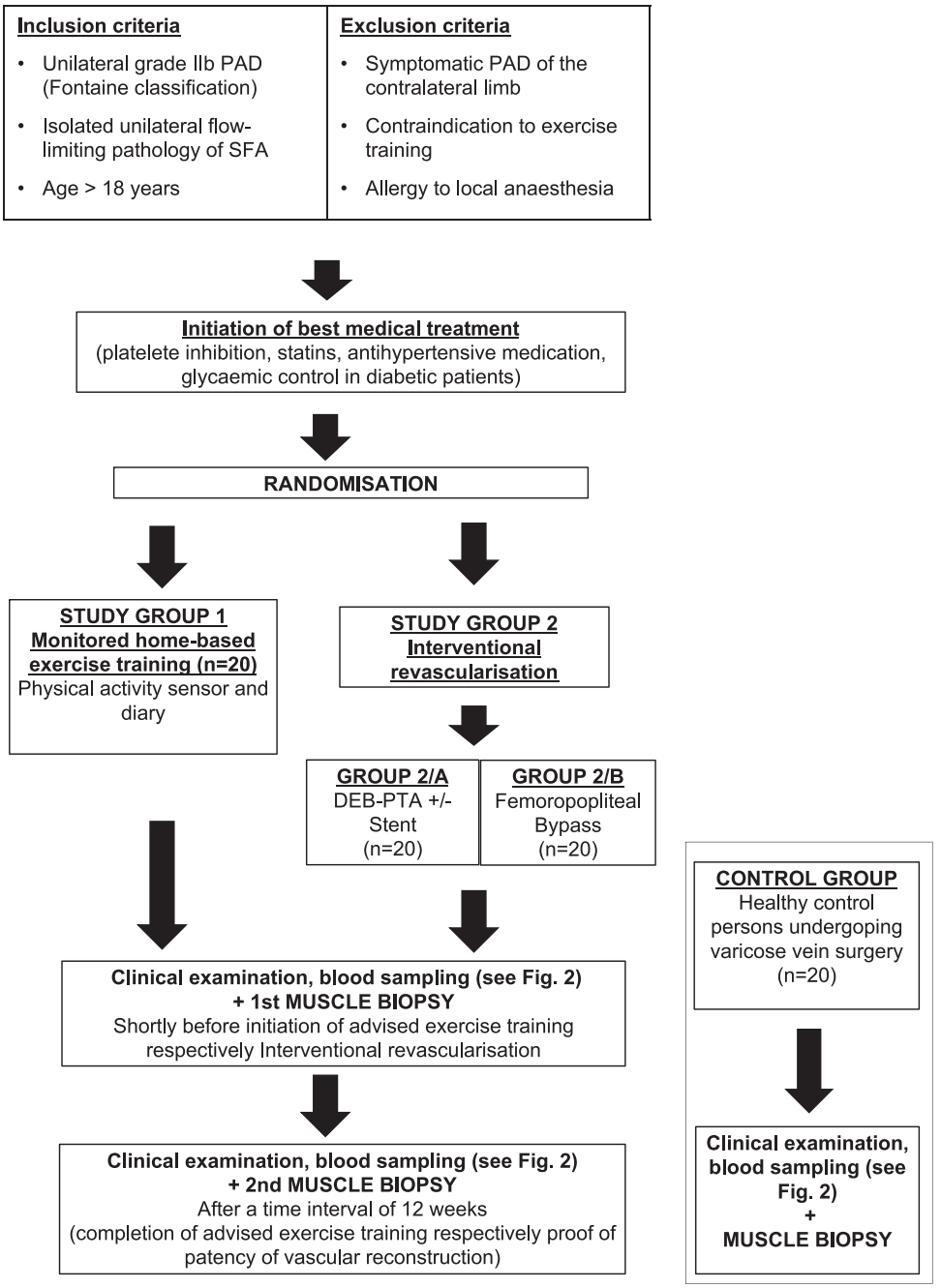

Fig. 1 – Study design. DEB-PTA = drug-eluting balloon percutaneous transluminal angioplasty; PAD = peripheral arterial disease; SFA = superficial femoral artery.

factors (smoking status, arterial hypertension, diabetes, and dyslipidaemia), history of previous cardiovascular diseases (myocardial infarction, PAD of the contralateral limb, and stroke), current medication with focus on anticoagulative and antiplatelet medication, as well as detailed medical history concerning intermittent claudication symptoms (location, duration, and character) will be obtained.

Clinical examination, blood sampling and in-vivo outcome analysis

All patients included in the study will undergo a defined sequence of clinical examinations as described in Figure 2. FMD of the brachial artery is the most widely used noninvasive method to evaluate endothelial dysfunction and is known to correlate with coronary artery endothelial dysfunction, as described by Andersson *et al.* when comparing coronary endothelial function testing to results from brachial artery measurements.[17,18] Details about the protocol used[19] are given in Supplement 2. Briefly, diameter and flow velocities of the brachial artery will be measured before and after the inflation of a cuff to a pressure >50% of systolic blood pressure being placed at the forearm, distally to the point of measurement. After the measurement of FMD, venous blood samples will be collected to study whether clinical differences can also be detected on a molecular level. Following the evaluation of pulses and the ABI on both lower extremities, study participants will undergo a standardized treadmill test (3 km per hour and 12% inclination) to evaluate the maximum walking distance. Before, during, and after the treadmill test, oxygen saturation on both calves will be monitored using continuous NIRS (Invos 7100, Covidien/Medtronic, Minneapolis, USA) according to the protocol described by Comerota *et al.* (see Supplement 3 for details). By the placement of NIRS probes on the patient's calves, information about oxygen delivery and demand during exercise will be provided.[20] After completion of the treadmill test, either because of walking distance limiting intermittent claudication or after a completed distance of 500 m, venous blood samples, pulse status, and measurement of ABI will be repeated. After resting for 30 min, clinical examinations will be completed by a standardized six-min walking test using the protocol by McDermott *et al.* (see Supplement 4 for details).[21] The described clinical examinations will be repeated after a time period of 12 wk for patients within the study groups.

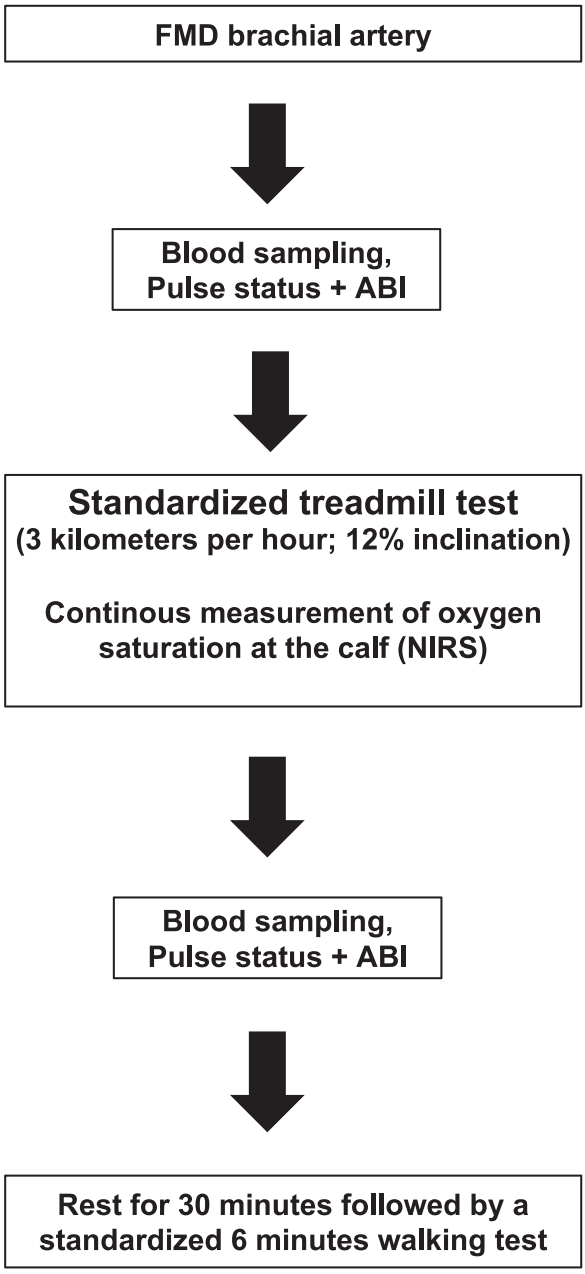

Fig. 2 – Sequence of clinical examinations. ABI = ankle brachial index; NIRS = near infrared refracted spectroscopy.

Muscle sampling for in-vitro evaluation of mitochondrial function

To enable evaluation of mitochondrial function by HRR and measurement of citrate synthase activity (CSA) in defined muscle regions muscle biopsies will be taken using the modified Bergstrom percutaneous biopsy technique.[22] Muscle biopsies will be obtained from both the ischemic muscle region (gastrocnemius muscle) and the non-ischemic muscle region (vastus lateralis muscle). Patients will be advised to rest for 15 min prior to the biopsy. Muscle biopsies will be obtained from the study groups prior to initiating treatment (exercise training/revascularization) as well as after 12 wk. In the control group, a muscle biopsy will be obtained once during varicose vein surgery. If muscle biopsies are taken during a surgical procedure, the samples will be taken under general anesthesia. For patients undergoing exercise training and for all patients during the second biopsy, samples will be collected under local anesthesia, using xylocaine. To avoid interference with mitochondrial function, no local anesthetic is infiltrated into the muscle.

About 5 mg of muscle tissue will be transferred to ice-cold biopsy preservation solution containing 10 mM Ca-ethylene

glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid buffer, 0.1 μM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM 2-(N-morpholino)-ethane-sulfonic acid hydrate, 0.5 mM dithiothreitol, 6.25 mM $MgCl_2$, 5.77 mM adenosine triphosphate, and 15 mM phosphocreatine (pH 7.1). The remaining muscle tissue will be mechanically permeabilized using a sharp angular forceps, resulting in separated muscle fibers that remain connected like a mesh-like framework. Permeabilization will be completed chemically by incubation of fibers in 2 mL biopsy preservation solution containing 50 μg/mL saponin for 30 min while being gently shaken on ice. Followed by a 10-min period of washing in a mitochondrial respiration medium (MiR06) containing 110 mM D-sucrose, 60 mM K^+-lactobionate, 0.5 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 g/L bovine serum albumin, and 280 U/ml catalase, muscle samples with a wet weight of 1 - 3 mg will be transferred to the respirometry chamber of the Oroboros O2k.[20]

HRR will be performed using an Oxygraph-2k (Oroboros Instruments, Innsbruck), as described in detail elsewhere,[23] and oxygen flux will be recorded using the DatLab Analysis Software (Oroboros Instruments, Innsbruck). To investigate mitochondrial respiration, substrates, uncouplers, and inhibitors of the respiratory chain will be titrated into the chambers (substrate-uncoupler-inhibitor titration-protocol) to induce a sequence of respiratory states. LEAK state, mitochondrial respiration when substrates of the respiratory chain are available, but adenosine diphosphate (ADP) is absent ($L_{(n)}$), will be induced by the titration of 2 mM of malate and 0.2 mM octanoylcarnitine ($MOct_L$). After stabilization of oxygen flux, 5 mM of ADP will be added to induce NADH-linked ADP-stimulated respiration. With the titration of 5 mM of pyruvate (substrate of the citric acid cycle) and 10 mM of succinate (direct substrate of complex II or succinate dehydrogenase), the OXPHOS-state (*P*) will be induced. Stepwise addition (0.05 mM steps) of the protonophore carbonyl cyanide p-(trifluoro methoxy) phenylhydrazone will yield maximum mitochondrial respiration or electron transfer capacity (*E*). Followed by the titration of 0.5 μM rotenone, an inhibitor of complex I, complex II-related respiration will be determined. With the final titration of 2.5 mM antimycin A, an inhibitor of the Q_i-binding site of complex III, residual oxygen consumption will be determined. Finally, the electron flow through Complex IV (cytochrome c oxidase) is measured by adding 2 mM ascorbate (As) and 0.5 mM tetramethyl-p-phenylenediamine.

About 10 mg of muscle tissue is initially stored at −80°C for further evaluation of CSA. After thawing, the samples will be homogenized, and protein concentrations of the lysates will be determined using a QuantiPro bicinchoninic acid Assay Kit (Sigma–Aldrich, St. Louis, MO, United States of America). CSA will be spectrophotometrically measured at 412 nm and 25°C using a commercial Citrate Synthase Assay Kit (Sigma–Aldrich) according to the manufacturer's instructions. The results from mass-specific mitochondrial respiration will be normalized to CSA in order to obtain mitochondrial-specific respiration expressed as pmol/(s*mg)/CSA.

Data collection and statistics

Data will be collected using pseudonymized case report forms and will be anonymized prior to statistical analysis. IBM SPSS (International Business Machines Corporation, Armonk, NY, USA) software will be used for statistical analysis. A sample size calculation was performed based on the existing literature about mitochondrial function in PAD patients and age-matched control persons,[24,25] assuming an effect size (Cohen's kappa) of 0.8. A probability of a type 1 error of 0.05 and power of 0.8 resulted in an adequate sample size of $n = 20$ per group. To compare values before and after 12 wk of treatment, a Wilcoxon single-rank test will be performed. To compare values between the study groups and the control group, a Mann–Whitney U test will be used. A *P* value of <0.05 will be statistically significant, while $P < 0.1$ will be considered a trend. Non-Gaussian distributed data will be expressed as median and interquartile range.

Discussion

The randomized controlled trial described here aims to elucidate the effect of different treatment modalities on mitochondrial function in symptomatic PAD. In a previous study, we found that the restoration of blood and nutrient supply had a greater impact on mitochondrial regeneration than increased physical activity. This was demonstrated by comparing mitochondrial alteration between ischemic and nonischemic muscle.[15] The impact of structured exercise training on overall mitochondrial function in ischemic muscle regions remained unclear. To clarify this, patients will be randomized to either structured, monitored home-based exercise training or a revascularization procedure using a similar study protocol regarding anatomic localization of atherosclerotic lesion and evaluation of mitochondrial alterations.[14,15] This will enable a differentiation of the mechanism leading to mitochondrial regeneration. The positive effect of exercise training on walking performance and quality of life in claudicants is indisputable.[26,27] It is known that a supervised setting is more effective than an unsupervized one.[28] To solve the problem of reimbursement for supervised exercise training, Gardner *et al.* initiated a randomized controlled trial comparing a home-based with a supervised one, with an additional usual care control group included. Patients assigned to home-based exercise training were provided with a physical activity monitor to document their training activities along with a detailed protocol.[29,30] The authors concluded that home-based exercise training is as effective as a standard supervised training program with additional benefits regarding daily ambulatory activity of patients.[29] Therefore, for this study, monitored home-based exercise training was chosen for the conservatively treated study group using the protocol described.[30] Mitochondrial alterations will be evaluated using HRR, which is widely recognized as the gold standard for assessing mitochondrial function.[31] In addition, citrate synthase activity will be measured to evaluate mitochondrial content. In this study protocol, additional in-vivo

application of noninvasive NIRS will enable the correlation of these methodologies to investigate mitochondrial function. NIRS is used to visualize the mismatch between oxygen demand and oxygen supply and is easily applicable before, during, and after exercise to locate the described mismatch distally of atherosclerotic lesions.[32] It is known that oxygen saturation within chronically ischemic muscle is reduced with NIRS being the noninvasive method of choice to detect these alterations.[20,33] NIRS can also detect mitochondrial oxidative capacity,[34] which allows for an overall mitochondrial assessment in the study population when correlated with established *in vivo* and *in vitro* methods.

PAD is known to be associated with an increased overall risk of cardiovascular mortality, with the ABI being a reliable indicator for this risk. An ABI lower than 0.90 or higher than 1.40 is associated with a higher risk of cardiovascular events.[35,36] Despite some reports about a decline of cardiovascular mortality in Western countries related to behavioral changes of patients and improvement in pharmacological treatment,[37] a meta-analysis of PAD outcomes found higher rates of all-cause and cardiovascular mortality in all reported PAD groups compared to a reference group.[7] The correlation of cardiovascular outcome parameters to mitochondrial function provides an integrative approach within this study protocol. In addition to the ABI, additional cardiovascular outcome parameters will be evaluated to further increase the validity. The vascular endothelium plays a crucial role in maintaining haemostasis and vascular health. Pathophysiological alterations in vascular tone, permeability, inflammation, and dedifferentiation can lead to the loss of haemostatic function of the endothelium.[17,18] The FMD has been defined as the most reliable noninvasive method for evaluating endothelial dysfunction and is strongly correlated with both coronary artery and peripheral artery endothelial (dys)function if applied.[17,18] Outcome parameters as well as the standardized walking test, which is also associated with overall cardiovascular health,[21,38] will provide a comprehensive evaluation of the enrolled patients and complement the results of the mitochondrial evaluation with an integrated research approach.

Cardiovascular diseases, such as heart failure, myocardial infarction, cardiomyopathies, hypertension, and atherosclerosis are associated with mitochondrial defects.[39,40] Therefore, there has been a strong effort over the last decades to develop new therapeutic targets that focus on mitochondrial dysfunction. One of the most intensively investigated cardiovascular pathology in regard to underlying mitochondrial function is heart failure.[41] Given the similarities between the (patho-) physiology of cardiomyocytes and skeletal muscle cells, the development of new targeted therapies in the field of heart failure is of interest for PAD as well. For example, coenzyme Q, being part of the electron transfer chain, is intensively investigated as a therapeutic target in the field of heart failure, as it is known that a reduced coenzyme Q pool might result in reverse electron transfer and reactive oxygen species generation.[42] An inverse correlation between the coenzyme Q plasma pool and mortality in heart failure was described as well.[43] Despite several placebo-controlled randomized trials trying to gain evidence for the beneficial and safe use of coenzyme Q in the therapy of heart failure, a systematic review, involving 11 studies and 1573 patients, concluded that there is still no convincing evidence to support or refute the use of coenzyme Q in patients with heart failure.[44]Still, investigating mitochondrial dysfunction in cardiovascular diseases, such as PAD, remains crucial to define new pathomechanisms and potential therapeutic targets.

In conclusion, this randomized controlled trial will provide further information on the effects of different treatment strategies on ischemic muscle regions in patients with symptomatic PAD. With an integrative study design, correlation of mitochondrial parameters to cardiovascular outcome parameters will enable a better understanding of mitochondrial dysfunction in PAD.

Disclosure

The authors declare that there is no conflict of interest.

Supplementary Materials

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jss.2025.01.014.

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Declaration of Generative AI and AI-Assisted Technologies in the Writing Process

The authors declare that no AI or AI-assisted technologies have been used in the writing process.

CRediT authorship contribution statement

Laura Schoenherr: Writing – original draft, Project administration, Methodology, Conceptualization. **Juliana Heidler:** Writing – original draft, Validation, Resources, Project administration, Methodology, Conceptualization. **Michaela Kluckner:** Writing – original draft, Project administration, Methodology, Conceptualization. **Daniela Lobenwein:** Writing – review & editing, Project administration, Methodology, Conceptualization. **Dominik Pesta:** Writing – review & editing, Supervision, Conceptualization. **Jan Paul Frese:** Writing – review & editing, Supervision, Methodology. **Sabine Helena Wipper:** Writing – review & editing, Supervision. **Alexandra Gratl:** Writing – original draft, Supervision, Project administration, Methodology, Conceptualization.

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